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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 48/00, A61P 35/00

A2

(11) International Publication Number: **WO 00/29033**

(43) International Publication Date: 25 May 2000 (25.05.00)

(21) International Application Number: PCT/US99/27206

(22) International Filing Date: 17 November 1999 (17.11.99)

(30) Priority Data:
60/108,881 17 November 1998 (17.11.98) US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

With a statement concerning non-prejudicial disclosure or exception to lack of novelty.

(54) Title: A METHOD OF GENETIC VECTOR DELIVERY

(57) Abstract

The invention relates to a method of administering a genetic vector to a target cell in a patient by immunosuppressing the patient and administering the genetic vector. The invention also relates to a method of administering a genetic vector to a target cell in a patient by administering a complement inhibitor and the genetic vector. The invention also relates to further administering a blood–organ barrier modifier.

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A Method of Genetic Vector Delivery

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Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

Background of the Invention

Field of the Invention

The invention relates to a method of administering a genetic vector to a target cell in a patient by immunosuppressing the patient, and administering the genetic vector. The invention relates to a method of administering a genetic vector to a target cell in a patient by inhibiting complement in the patient, and administering the genetic vector. The invention relates to a method of administering a genetic vector to a target cell in a patient by immunosuppressing the patient, and administering a blood-organ barrier modifier and the genetic vector. The invention also relates to a method of administering a genetic vector to a brain cell in a patient by immunosuppressing the patient, and administering an agent which increases blood volume in the brain of the patient and the genetic vector. The invention further relates to a method of administering a genetic vector to a target cell in a patient by immunosuppressing the patient, and administering a blood-brain barrier modifier and the genetic vector.

Related Art

Non-viral and viral vectors have been exploited to treat or prevent diseases, including cancer, in animals and humans (*see*, Desnick, R. and Schuchman, E.H., *Acta Paediatrica Japonica 40*:191-203 (1998); Robbins, P.D. *et al., Tibtech 16*:35-40 (1998)). Non-viral vectors used for gene therapy include,

for example, plasmid DNA, liposome-entrapped DNA, and protein-DNA conjugates.

Viral vector therapies have included two distinct approaches: (i) direct cell killing (oncolysis) by a mutagenized virus, called oncolysis (Martuza *et al.*, *Science 252*:854-856 (1991); Mineta *et al.*, *Nature Med 1*:938-943 (1995); Boviatsis *et al.*, *Cancer Res. 54*: 5745-5751 (1994); Kesari, *et al.*, *Lab. Invest. 73*: 636-648 (1995); Chambers *et al.*, *Proc. Natl. Acad. Sci. USA 92*: 1411-1415 (1995); Lorence, R.M. *et al.*, *J. Natl. Cancer. Inst. 86*: 1228-1233 (1994); Bischoff, *et al.*, *Science 274*: 373-376 (1996); Rodriguez *et al.*, *Cancer Res. 57*: 2559-2563 (1997)), and (ii) the use of viral vectors to deliver a transgene whose expression product corrects a defect which causes a disease or activates a chemotherapeutic agent (Wei *et al.*, *Human Gene Therapy 5*: 969-978 (1994); Chen and Waxman, *Cancer Res. 55*: 581-589 (1995); Moolten, *Cancer Gene Ther. 1*: 279-287 (1994); Fakhrai *et al.*, *Proc. Natl. Acad. Sci. USA 93*: 2909-2914 (1996); Roth *et al.*, *Nature Med. 2*: 985-991 (1996); Moolten, *Cancer Res. 46*: 5276-5281 (1986); Chen *et al.*, *Proc. Natl. Acad. Sci. USA 91*: 3054-3057 (1994)).

With regard to the first approach in viral vector therapy, viral oncolysis for cancer treatment, genetic engineering of viruses has focused on generating "replication-conditional" viruses that are not capable of replication in non-dividing cells in order to avoid systemic infection. Viruses capable of replication in dividing cells preferentially infect rapidly dividing tumor cells because they are incapable of replicating in non-dividing normal cells.

Viral vectors have been created having mutations in selected adenoviral or herpes simplex virus type 1 genes, in order to render viral replication conditional for tumor cells. For example, an adenovirus with a deletion in the E1B-55Kd encoding gene has been shown to selectively replicate in p53-defective tumor cells (Bischoff, *et al.*, *Science 274*: 373-376 (1996)). Herpes simplex viruses type 1 (HSV-1) with deletions or insertions in viral genes encoding for thymidine kinase (Martuza *et al.*, *Science 252*:854-856 (1991)) (Hstk), ribonucleotide reductase

(Hsrr) (Mineta *et al., Nature Med. 1*: 938-943 (1995); Boviatsis *et al., Cancer Res. 54:* 5745-5751 (1994)), or $\gamma_{34.5}$ (Mineta *et al., Nature Med 1*:938-943 (1995); Chambers *et al., Proc. Natl. Acad. Sci. USA 92*: 1411-1415 (1995)), have also been shown to replicate in and lyse dividing cells but not quiescent cells, presumably because the former can complement the defective viral function (Goldstein and Weller, *J. Virol. 62*: 196-205 (1988)). *See also*, Kesari *et al., Lab. Invest. 73*: 636-648 (1995); Lorence, R.M. *et al., J. Natl. Cancer. Inst. 86*: 1228-1233 (1994); Rodriguez *et al., Cancer Res. 57*: 2559-2563 (1997).

Oncolytic viruses, such as the herpes simplex viral mutant hrR3, have shown evidence of anticancer effects in experimental brain tumor models (Martuza, R. L., *et al., Science 252*:854-856 (1991); Boviatsis, E. J., *et al., Cancer Res. 54*:5745-5751 (1994); Boviatsis, E. J., *et al., Gene Therapy 1*:323-331 (1994); Mineta, T., *et al., Nature Med 1*:938-943 (1995); Kramm, C.M., *et al., Hum. Gene. Ther. 7*:291-300 (1996); Andreansky, S., *et al., Cancer Res. 57*:1502-1509 (1997); Pyles, R. B., *et al., Hum. Gene Ther. 8*:533-544 (1997); Kramm, C. M., *et al., Hum. Gene Ther. 8*:2057-2068 (1997); Rainov, N. G., *et al., Cancer Gene Therapy 5*:158-162 (1998)).

As mentioned above, a second approach in viral vector therapy is the viral delivery of a transgene to correct a defect or anticancer transgenes (Wei *et al., Human Gene Therapy 5*: 969-978 (1994); Chen and Waxman, *Cancer Res. 55*: 581-589 (1995); Moolten, *Cancer Gene Ther. 1*: 279-287 (1994); Fakhrai *et al., Proc. Natl. Acad. Sci. USA 93*: 2909-2914 (1996); Roth *et al., Nature Med. 2*: 985-991 (1996); Moolten, *Cancer Res. 46*: 5276-5281 (1986); Chen *et al., Proc. Natl. Acad. Sci. USA 91*: 3054-3057 (1994); Mroz, and Moolten, *Hum. Gene Ther. 4*: 589-595 (1993); Mullen *et al., Proc. Natl. Acad. Sci. USA 59*: 33-37 (1992); Wei *et al., Clin. Cancer Res. 1*: 1171-1177 (1995); Marais *et al., Cancer Res. 56*: 4735-4742 (1996); Chen *et al., Cancer Res. 56*: 1331-1340 (1996)).

Although intratumoral inoculation of oncolytic viruses into the tumor mass can provide control of tumor growth and, in some instances, can lead to extensive tumor regression, it is likely that the volumetric extent of viral penetration into the

tumor mass may still be limited. This may be even more true in the case of multicentric foci of tumor present in different regions of the brain, since the oncolytic virus inoculated into one site is unlikely to spread to a second separate site. One possible solution to this predicament is to employ an intravascular route of delivery of the oncolytic viral vector. In previous studies, an intra-arterial injection route was used to deliver an oncolytic HSV or adenoviral vector into rodent models of brain tumors; the blood-brain barrier required disruption by pharmacologic or osmotic means (Nilaver, G., *et al., Proc. Natl. Acad. Sci. 92*:9829-9833 (1995); Rainov, N.G., *et al., Human Gene Ther. 6*:1543-1552 (1995)).

The blood-brain barrier (BBB) is a single layer of brain capillary endothelial cells that are bound together by tight junctions. The BBB excludes entry of many blood-borne molecules, particularly viruses. Nilaver, G. *et al., Proc. Natl. Acad. Sci. USA 92*:9829-9833 (1995), found that osmotic BBB disruption allowed transvascular delivery of viral vectors to intracerebral tumor when administered intraarterially. Disruption of the blood-brain barrier by intra-carotid bradykinin infusion was found to enhance HSV delivery into brain tumors (Rainov, N.G. *et al., Human Gene Therapy 6*:1543-1552 (1995); Rainov, N.G. *et al., Cancer Gene Ther. 5*:158-162 (1998)). RMP-7, a synthetic peptidergic bradykinin agonist was reported to increase the permeability of the blood-brain barrier by opening the tight junctions between the endothelial cells of brain capillaries (Elliott, P.J. *et al., Exptl. Neurol. 141*:214-224 (1996)). RMP-7 is a bradykinin B2 receptor agonist, which increases permeability of the BBB, especially associated with brain tumors (Bartus, R.T. *et al., Immunopharm. 33*:270-278 (1996); Bartus, R.T. *et al., Exptl. Neurol. 142*:14-28 (1996)). RMP-7 was reported to enhance the uptake of ganciclovir (GCV) across the blood-brain tumor barrier in rats bearing C6 intracerebral gliomas, resulting in reduced tumor growth (LeMay, D.R. *et al., Human Gene Therapy 9*:989-995 (1998)).

In the process of attempting to understand anticancer effects, it has been discovered that intra-arterial delivery of an oncolytic virus can lead to extensive

transduction of relatively large and pre-existing tumors established in the brains of rodents and thus result in long-term survivorship. Successful achievement of this result requires the combination of pharmacologic disruption of the blood-brain barrier and immunosuppression. This is surprising because the current thought in anticancer gene therapy is that an active immune system is necessary for effective tumor regression (Vile, R.G. *et al., Cancer Res. 54*:6228-6234 (1994); Mullen, C.A. *et al., Cancer Res. 54*:1503-1506 (1994); and Chen, S.-H. *et al., Cancer Res. 56*:3758-3762 (1996)).

Summary of the Invention

The invention is directed to a method of administering a genetic vector to a target cell in a patient, the method comprising: (a) immunosuppressing the patient; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient a blood-organ barrier modifier in a pharmaceutically effective amount to disrupt a blood-organ barrier in the patient. The invention is directed to further (d) administering a complement inhibitor to the patient.

The invention is also directed to a method of administering a genetic vector to a target cell in a patient, the method comprising: (a) administering a complement inhibitor; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient a blood-organ barrier modifier in a pharmaceutically effective amount to disrupt a blood-organ barrier in the patient.

The method can be for treatment of, but not limited to, a neoplasm. The neoplasm is a tumor. The tumor can be of the central nervous system (CNS) or metastasized to the CNS. The tumor includes, but is not limited to, astrocytoma, oligodendroglioma, neurofibroma, glioblastoma, ependymoma, Schwannoma, neurofibrosarcoma, meningioma, medulloblastoma, and metastatic cancer. The

tumor can be a brain tumor. The brain tumor includes, but is not limited to, astrocytoma, oligodendroglioma, and glioblastoma.

The target cell includes a brain cell, eye cell, testicular cell, and nerve cell. The target cell can also be an endothelial cell, ependymal cell, glial cell and neuron. The target cell can be a neoplastic cell or malignant or neoplastic cells which have metastasized to the brain, eye, testis or nervous system.

The patient can be a non-human animal or a human.

The method can selectively kill a neoplastic cell. The method can lyse the neoplastic cell.

In the invention, the patient can be immunosuppressed by, for example, administering a pharmaceutically effective amount of an agent selected from the group consisting of azathioprine, cobra venom factor, cyclosporin A, FK506, FK520 and ganciclovir. The patient can be immunosuppressed by administering, for example, a pharmaceutically effective amount of an agent selected from the group consisting of cyclophosphamide, ifosfamide and 5-fluorouracil.

The blood-organ barrier modifier can be administered intravascularly. The blood-organ barrier can be a blood-brain barrier modifier. A blood-brain barrier modifier can be a blood-brain-tumor barrier modifier. Thus, a blood-organ barrier modifier can be a blood-brain-tumor barrier modifier. Blood-organ barrier modifiers include, but are not limited to, bradykinin agonists, osmotic agents, cyclic GMP modulators, cytokines, nitric oxide modulators, by physical methods, and combinations thereof. Preferred blood-organ barrier modifiers are bradykinin agonists, preferably bradykinin, and most preferably, RMP-7.

The osmotic agent can be, for example, mannitol or urea.

The cyclic GMP modulator can be, but is not limited to, dimaprit or zaprinast.

The cytokine can be, for example, interleukin-1β, tumor necrosis factor-α or interferon-γ.

The nitric oxide modulator can be, but is not limited to, L-arginine, sodium nitroprusside, SIN-T(3-morpholinosydnone imine), diethylamine/NO,

diethylenetriamine/NO, spermine/NO, S-nitrous-N-acetyl cysteine or proline-nitric-oxide.

The physical method can be, for example, radiation or focused ultrasound.

In the invention, the genetic vector can be administered intravascularly. The genetic vector can be a viral vector or a non-viral vector. The viral vector can be derived from, but is not limited to, herpes virus, adenovirus, retrovirus, lentivirus, cytomegalovirus, varicella zoster virus, psuedorabies, adeno-associated virus, Epstein-Barr virus, or hybrid virus. A preferred viral vector is derived from herpes simplex virus.

The non-viral vector can be, but is not limited to, a liposome vector, gene "gun" vector, or plasmid vector.

The genetic vector can contain a gene encoding a gene product capable of converting a chemotherapeutic agent to its cytotoxic form, wherein the chemotherapeutic agent does not significantly inhibit replication of the genetic vector.

The complement inhibitor can be, but is not limited to, soluble complement receptor 1, soluble complement receptor 1 deleted in C4 binding region, antibody against C5, specific isocoumarin inhibitor of factor B and factor D, inhibitor of C2, C1 inhibitor, and SDZGPI 562, SUT 175, and K76.

The invention is directed to a method of administering a genetic vector to a brain cell in a patient, the method comprising: (a) immunosuppressing the patient; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient an agent, which increases blood volume in brain of the patient, in a pharmaceutically effective amount to disrupt a blood-brain barrier in the patient. The invention is directed to further (d) administering a complement inhibitor to the patient.

The invention is also directed to a method of administering a genetic vector to a brain cell in a patient, the method comprising: (a) administering a complement inhibitor; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient an agent, which

increases blood volume in brain of the patient, in a pharmaceutically effective amount to disrupt a blood-brain barrier in the patient.

The method can be for treatment of, but not limited to, a brain tumor. In the invention, the brain cell can be a brain tumor cell or malignant or neoplastic cells which have metastasized to the brain. The brain tumor includes, but is not limited to, astrocytoma, oligodendroglioma, and glioblastoma.

The patient can be a non-human animal or a human.

The method can selectively kill the brain tumor cell. The method can lyse the brain tumor cell.

In the invention, the patient can be immunosuppressed by, for example, administering a pharmaceutically effective amount of an agent selected from the group consisting of azathioprine, cobra venom factor, cyclosporin A, FK506, FK520 and ganciclovir. The patient can be immunosuppressed by administering, for example, a pharmaceutically effective amount of an agent selected from the group consisting of cyclophosphamide, ifosfamide and 5-fluorouracil. Cyclophosphamide is a preferred immunosuppressant.

The agent which increases blood volume in brain of a patient can be administered intravascularly. The agent can be a blood-brain barrier modifier, including a blood-brain-tumor barrier modifier. Agents which increase blood volume in brain of a patient can include, but are not limited to, bradykinin agonists, osmotic agents, cyclic GMP modulators, cytokines, nitric oxide modulators, by physical methods, and combinations thereof. Preferred agents are bradykinin agonists, preferably bradykinin, and most preferably, RMP-7.

The osmotic agent can be, for example, mannitol or urea.

The cyclic GMP modulator can be, but is not limited to dimaprit or zaprinast.

The cytokine can be, for example, interleukin-1β, tumor necrosis factor-α or interferon-γ.

The nitric oxide modulator can be, but is not limited to, L-arginine, sodium nitroprusside, SIN-T(3-morpholinosydnone imine), diethylamine/NO,

diethylenetriamine/NO, spermine/NO, S-nitrous-N-acetyl cysteine or proline-nitric-oxide.

The physical method can be, for example, radiation or focused ultrasound.

In the invention, the genetic vector can be administered intravascularly. The genetic vector can be a viral vector or a non-viral vector. The viral vector can be derived from, but is not limited to, herpes virus, adenovirus, retrovirus, lentivirus, cytomegalovirus, varicella zoster virus, psuedorabies, adeno-associated virus, Epstein-Barr virus, or hybrid virus. A preferred viral vector is derived from herpes simplex virus.

The non-viral vector can be, but is not limited to, a liposome vector, gene "gun" vector, or plasmid vector.

The genetic vector can contain a gene encoding a gene product capable of converting a chemotherapeutic agent to its cytotoxic form, wherein the chemotherapeutic agent does not significantly inhibit replication of the genetic vector.

The complement inhibitor can be, but is not limited to, soluble complement receptor 1, soluble complement receptor 1 deleted in C4 binding region, antibody against C5, specific isocoumarin inhibitor of factor B and factor D, inhibitor of C2, C1 inhibitor, and SDZGPI 562, SUT 175, and K76.

The invention is directed to a method of administering a genetic vector to a target cell in a patient, the method comprising: (a) immunosuppressing the patient; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient a blood-brain barrier modifier in a pharmaceutically effective amount to disrupt a blood-brain barrier in the patient. The invention is directed to further (d) administering a complement inhibitor to the patient.

The invention is also directed to a method of administering a genetic vector to a target cell in a patient, the method comprising: (a) administering a complement inhibitor; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient a blood-brain

barrier modifier in a pharmaceutically effective amount to disrupt a blood-brain barrier in the patient.

The method can be for treatment of diseases such as, for example, an inborn error of metabolism, neurodegenerative disorder, stroke, tumor, spinal cord trauma, and nerve trauma. The inborn error of metabolism can be, for example, Tay-Sachs disease. Neurodegenerative disorders include, but are not limited to, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis.

The target cell can a brain cell or malignant or neoplastic cells which have matastasized to the brain. The patient can be a non-human animal or a human.

The method can selectively kill a neoplastic cell. The method can lyse the neoplastic cell.

In the invention, the patient can be immunosuppressed by, for example, administering a pharmaceutically effective amount of an agent selected from the group consisting of azathioprine, cobra venom factor, cyclosporin A, FK506, FK520 and ganciclovir. The patient can be immunosuppressed by administering, for example, a pharmaceutically effective amount of an agent selected from the group consisting of cyclophosphamide, ifosfamide and 5-fluorouracil. A preferred method of immunosuppressing a patient is by administering cyclophosphamide.

The blood-brain barrier modifier can be administered intravascularly. The blood-brain barrier modifier can be a blood-brain-tumor barrier modifier. Exemplary blood-brain barrier modifiers include, but are not limited to, bradykinin agonists, osmotic agents, cyclic GMP modulators, cytokines, nitric oxide modulators, by physical methods, and combinations thereof. Preferred blood-brain barrier modifiers are bradykinin agonists, preferably bradykinin, and most preferably, RMP-7.

The osmotic agent can be, for example, mannitol or urea.

The cyclic GMP modulator can be, but is not limited to dimaprit or zaprinast.

The cytokine can be, for example, interleukin-1β, tumor necrosis factor-α or interferon-γ.

The nitric oxide modulator can be, but is not limited to, L-arginine, sodium nitroprusside, SIN-T(3-morpholinosydnone imine), diethylamine/NO, diethylenetriamine/NO, spermine/NO, S-nitrous-N-acetyl cysteine or proline-nitric-oxide.

The physical method can be, for example, radiation or focused ultrasound.

In the invention, the genetic vector can be administered intravascularly. The genetic vector can be a viral vector or a non-viral vector. The viral vector can be derived from, but is not limited to, herpes virus, adenovirus, retrovirus, lentivirus, cytomegalovirus, varicella zoster virus, psuedorabies, adeno-associated virus, Epstein-Barr virus, or hybrid virus. A preferred viral vector is derived from herpes simplex virus.

The non-viral vector can be, but is not limited to, a liposome vector, gene "gun" vector, or plasmid vector.

The complement inhibitor can be, but is not limited to, soluble complement receptor 1, soluble complement receptor 1 deleted in C4 binding region, antibody against C5, specific isocoumarin inhibitor of factor B and factor D, inhibitor of C2, C1 inhibitor, and SDZGPI 562, SUT 175, and K76.

Brief Description of the Figures

Figure 1. Intravascular administration of oncolytic virus to a single human U87dEGFR glioma xenograft in rodent brain. **Figure 1A:** LacZ gene transduction and "plaque" formation in the tumor two days after treatment with hrR3, RMP-7 and CPA (panels 1, 2 and 3), hrR3 alone (panel 4), hrR3 and RMP-7 (panel 5), or hrR3 and CPA (panel 6). In panel 3, a section adjacent to the section shown in panel 2, was immunohistochemically processed using polyclonal antiserum raised against HSV thymidine kinase. Scale bars=200 μm. **Figure 1B:** Kaplan-Meier survival curves. The arrow indicates time point of catheterization. **Figure 1C:** CPA inhibits the viral neutralizing activity of athymic rat plasma. Plasma was prepared from animals at day 0, 2, 4, and 8 after injection

with hrR3, RMP-7 and saline (open circles) or CPA (closed circles), and titers of neutralizing antibodies against HSV were assayed.

Figure 2. Intravascular administration of oncolytic virus to three separate human glioma xenografts in rodent brain. **Figure 2A:** LacZ gene transduction within three separate human glioma xenografts. Two representative sections from one brain harboring the three tumors positively transduced by the oncolytic virus are shown 2 (panels 1 and 4) and 4 (panels 2 and 5) days after administration of CPA, hrR3, and RMP-7. Tiny residual tumors (panel 3) or complete disappearance of tumor (panel 6) were seen 8 days after intravascular injection. (Bar: 2 mm). **Figure 2B:** Kaplan-Meier survival curves. The arrow indicates time point of catheterization.

Figure 3. Presence of an innate antiviral activity in rodent plasma that is CPA-and heat-labile. Plasma was prepared from immunocompetent (Figs. 3A, 3C, and 3E) and athymic (Figs. 3B, 3D, and 3F) rats, 2 days after intraperitoneal CPA (or carrier) administration. Figs. 3A and 3B, serial dilutions of heated plasma were employed in the inactivation assay. Figs. 3C and 3D, heated and undiluted plasma was added onto Vero cells in culture, washed off the cells before assaying for hrR3 activity. Figs. 3E and 3F, plasma was prepared before (day 0) or 2 days after intravascular administration of hrR3 (or mock). For the experiment shown in panel f, undiluted athymic rat plasma is used. The low values in the Y-axis (compared to those of Fig. 3E) reflect the more potent antiviral activity of undiluted athymic rat plasma, and correspond to the almost invisible first two bars in Fig. 3B.

Figure 4. The innate antiviral activity is also present in human plasma. Human plasma was collected from patients, before and after they underwent chemotherapy with CPA. Heat-treated (Fig. 4A), and unheated (Fig. 4B) plasma was employed in the inactivation assay.

Figure 5. The innate antiviral activity interacts with complement in a calcium dependent manner. Plasma was prepared from immunocompetent (Fig. 5A) and athymic (Fig. 5B) rats that had been injected intraperitoneally with CPA (or carrier) on day -2 and/or with cobra venom factor (or carrier) on day -1 at a dose of 60 U/kg and on day 0 at 20 U/kg (days numbered with respect to the day of sacrifice). Serial dilutions of unheated plasma were employed in the inactivation assay. Plasma was also treated with EGTA-Mg before the assay. Note that at the 1:8 dilution innate antiviral action abrogates viral survival, with very little effect from CPA. However, pre-treatment with CVF or EGTA-Mg reverses partially (for CVF) or completely (for EGTA-Mg) the antiviral effect of 1:8 diluted, un-heated plasma. The addition of CPA results in an even greater reversal.

Figure 6. Pretreatment of rodent plasma with rabbit anti-rat IgM abrogates the innate antiviral activity in a dose-dependent fashion. In Fig. 6A, harvested rodent plasma was pre-incubated with increasing doses of rabbit anti-rat IgM (closed circles) or rabbit IgG (open circles) before incubating with hrR3. Virus survival was measured in a lacZ-plaque neutralization assay. The x-axis is in log scale. In Fig. 6B, plasma (un-heated and diluted 1:8) harvested from rodents treated or not treated with CPA was pre-incubated with 60 mg/dl of rabbit anti-rat IgM or vehicle before incubation with hrR3.

Figure 7. Plasma anti-HSV activity and partial reversal by cobra venom factor (CVF). Plasma was prepared from athymic rats 48 hours days after intraperitoneal administration of CVF (or carrier), as detailed in the Methods section. After serially diluting it, it was mixed with oncolytic HSV before adding onto Vero cells. Percent virus survival denotes the percent of lacZ-expressing Vero cells, enumerated 16 hours after infection. The bars represent the average from triplicate dishes and error bars represent the standard error of the mean.

Figure 8. Effects of CVF-treated and control plasma on three HSV vectors and adenovirus. Plasma was prepared from athymic rats, two days after treatment with CVF or carrier. It was then diluted 1:8, before mixing with hrR3, an ICP6-defective HSV derived from KOS strain, MGH1, an ICP6- and ICP34.5-defective HSV derived from F strain, Amplicon (Ampl.), a replication defective HSV amplicon derived from strain 17, or an Adenoviral (Ad.) vector. Percent virus survival denotes the percent of *lacZ*-expressing Vero cells, enumerated 16 hours after infection. The bars represent the average from triplicate dishes and error bars represent the standard error of the mean.

Figure 9. Rat complement inactivates oncolytic RSV. The hrR3 mutant virus was pre-incubated with HBSS, purified rat complement (3 mg/ml in HBSS), designated by C, heat-inactivated rat plasma (diluted 1:4) designated by HI, or rat complement re-added to heat-inactivated plasma designated by C + HI, for 1.5 hours before adding onto Vero cells in culture. Percent virus survival denotes the percent of lacZ-expressing Vero cells (enumerated 16 hours after infection) infected with hrR3 treated with HBSS. The bars represent the average from triplicate dishes and error bars represent the standard error of the mean.

Figure 10. Histologic sections of brains with three neoplasms, two (panel A), four (panel B) and eight (panel C) days after intravascular treatment with hrR3, in the presence or absence of CVF and CPA. Human U87dEGFR glioma cells were implanted into three separate intracerebral locations (right and left frontal lobes and right thalamus). Animals were treated with CVF (60 U/kg) or saline, six days later, and with another dose of CVF (20 U/kg) or saline, seven days later. At this time point, animals were treated with intravascular hrR3 and RMP-7. Some animals also received an intraperitoneal injection of CPA (100 mg/kg), two days after intravascular treatment with hrR3. Animals were sacrificed two (panel A), four (panel B), and eight days (panel C) after virus administration. Two days later, some of them were sacrificed and their brains were harvested,

sectioned, and stained for *lacZ* cDNA expression. The anatomic extent of tumor transduction was measured for each of three neoplasms and tabulated in Tables 3 and 4. Here, photomicrographs of sections showing brains with tumors stained for *lacZ* cDNA expression reveals transgene expression in a "plaque"-like configuration within tumors from CVF treated-animals, two days after administration of hrR3 (arrow in panel A). Four days later, the anatomic area of the lacZ-expressing "plaques" has not increased in the CVF-treated animals. However, the addition of CPA results in a significant augmentation of lacZ-expressing cells within the tumors (Panel B). By eight days, the oncolytic action of the virus has resulted in tumor involution (Arrows in panel C). Bars = 4 min.

Figure 11. Kaplan-Meier survival analyses of athymic rats harboring three separate human glioma xenografts. Fig. 11A, survival of rats injected with hrR3. Fig. 11B, survival of rats injected with mock. Arrows indicate the time point of catheterization. In the hrR3-treated group, the differences in survival between animals treated with CVF + CPA versus CVF alone or CPA alone were both statistically significant ($P < 0.001$, Wilcoxon signed rank test). The addition of RMP7 to the hrR3 + CVF+ CPA treatment produced a slight increase in survival, but was not statistically significant ($P=0.2$). All treatments with hrR3 were significant when compared to treatments without virus ($P<0.00\ 1$).

Figure 12. Analysis of viral genomes in athymic rat tissues after intra-arterial administration of hrR3 in the presence of CVF and CPA. Genomic DNA was prepared from athymic rats' brain tumor (lane 2), brain surrounding tumor (lane 3), lung (lane 4), liver (lane 5), spleen (lane 6), and kidney (lane 7) and was then analyzed by the polymerase chain reaction (PCR) using primers specific for hrR3. The 5'-primer hybridizes to the 5'-region of the HSV ICP6 genome and the 3'-primer hybridizes to the 5'-region of the inserted lacZ cDNA. After agarose gel electrophoresis and ethidium bromide staining (shown in the panel on the left), Southern analysis of the PCR products was performed using a

lacZ cDNA probe that should hybridize to the amplified PCR fragments. The size of the PCR product is approximately 1000 base pairs.

Detailed Description of the Preferred Embodiments

The invention is directed to a method of administering a genetic vector to a target cell in a patient, the method comprising: (a) immunosuppressing the patient; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient a blood-organ barrier modifier in a pharmaceutically effective amount to disrupt a blood-organ barrier in the patient. The invention is directed to further (d) administering a complement inhibitor to the patient.

The invention is also directed to a method of administering a genetic vector to a target cell in a patient, the method comprising: (a) administering a complement inhibitor; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient a blood-organ barrier modifier in a pharmaceutically effective amount to disrupt a blood-organ barrier in the patient.

The invention is directed to a method of administering a genetic vector to a brain cell in a patient, the method comprising: (a) immunosuppressing the patient; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient an agent, which increases blood volume in brain of the patient, in a pharmaceutically effective amount to disrupt a blood-brain barrier in the patient. The invention is directed to further (d) administering a complement inhibitor to the patient.

The invention is also directed to a method of administering a genetic vector to a brain cell in a patient, the method comprising: (a) administering a complement inhibitor; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient an agent, which

increases blood volume in brain of the patient, in a pharmaceutically effective amount to disrupt a blood-brain barrier in the patient.

The invention is directed to a method of administering a genetic vector to a target cell in a patient, the method comprising: (a) immunosuppressing the patient; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient a blood-brain barrier modifier in a pharmaceutically effective amount to disrupt a blood-brain barrier in the patient. The invention is directed to further (d) administering a complement inhibitor to the patient.

The invention is also directed to a method of administering a genetic vector to a target cell in a patient, the method comprising: (a) administering a complement inhibitor; and (b) administering the genetic vector to the patient. The invention is directed to further (c) administering to the patient a blood-brain barrier modifier in a pharmaceutically effective amount to disrupt a blood-brain barrier in the patient.

Neoplasia is a process by which the normal controlling mechanisms that regulate cell growth and differentiation are impaired resulting in progressive growth. During neoplasia, there is a characteristic failure to control cell turnover and growth. This lack of control causes a tumor to grow progressively, enlarging and occupying spaces in vital areas of the body. If the tumor invades surrounding tissue and is transported to distant sites the tendency of this tumor will be to result in death of the individual.

By "neoplastic cells" is intended cells whose normal growth control mechanisms are disrupted (typically by accumulated genetic mutations), thereby providing potential for uncontrolled proliferation. Thus, "neoplastic cells" can include both dividing and non-dividing cells.

By a "target cell" is intended cells which the genetic vector is intended to act on or affect. In the invention, the target cell includes a brain cell, eye cell, testicular cell, and nerve cell. The target cell can also be an endothelial cell, ependymal cell, glial cell and neuron. The target cell can be a neoplastic cell or

malignant or neoplastic cells which have metastasized to the brain, eye, testis or nervous system. Preferred target cells of the invention are tumor cells, especially brain tumor cells.

The invention can be utilized to target, for oncolysis, both benign and malignant neoplastic cells and the brain. For purposes of the invention, neoplastic cells include cells of tumors, neoplasms, carcinomas, sarcomas, leukemias, lymphomas, and the like. Of particular interest in the invention are central nervous system (CNS) tumors (*see*, Chung, R.Y. and Chiocca, E.A., *Cancer Gene Therapy* 7:589-602 (1998)). These include astrocytomas, oligodendrogliomas, meningiomas, neurofibromas, ependymomas, Schwannomas, neurofibrosarcomas, glioblastomas, medulloblastoma, metastatic cancer, etc. The neoplastic cells of particular concern to the invention are those cells of brain tumors. Exemplary brain tumors include, but are not limited to, astrocytoma, oligodendroglioma, and glioblastoma.

By "immunosuppressing" is intended preventing or interfering with the immunologic response of a patient by, for example, suppressing the release of cytokines, suppressing maturation of B-cell responses, suppressing T-cell responses, depleting T-cells, etc. Agents that cause immunosuppression, or "immunosuppressants," and dosages required for immunosuppression are well known in the art (*see*, Goodman and Gilman, *The Pharmacological Basis of Therapeutics*, Pergamon Press, 9th ed. (1996)). Of course, the dosage can vary depending on the subject to be treated. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. Exemplary immunosuppressants include, but are not limited to, azathioprine, cobra venom factor, cyclosporin A, FK506, FK520, ganciclovir, cyclophosphamide, ifosfamide and 5-fluorouracil. A preferred immunosuppressant is cyclophosphamide. Wagoner, L.E. *et al., Clin. Transplantation 10*:437-443 (1996), discusses the use of cyclophosphamide as an immunosuppressive.

By "inhibiting complement" is intended inhibiting or depleting any components belonging to the complement pathway. It is known in the art that complement is a complex series of some 20 proteins which, along with blood clotting, fibrinolysis and kinin formation, forms one of the triggered enzyme systems found in plasma. These systems produce a rapid, highly amplified response to a trigger stimulus mediated by a cascade phenomenon where the product of one reaction is the enzymic catalyst of the next. Complement inhibitors include, but are not limited to, soluble complement receptor 1 (Weisman, H.F. *et al., Science 249*:146-151 (1990)), soluble complement receptor 1 deleted in C4 binding region (Gralinski, M.R. *et al., Immunopharmacology 34*:79-88 (1996); Scesney, S.M. *et al., Eur. J. Immunol. 26*:1729-35 (1996)), antibody against C5 (Czermak, B.J. *et al., Nat Med. 5*:788-792 (1996)), specific isocoumarin inhibitor of factor B and factor D (Kam, C.M. *et al., J. Immunol. 149*:163-168 (1992)), inhibitor of C2 (Kroes, B.H. *et al., Immunology 90*:115-120 (1997)), C1 inhibitor (Fischer, M.B. *et al., J. Immunol. 159*:976-982 (1997)), and SDZGPI 562, SUT 175, and K76 (Blum, M.G. *et al., Xenotransplantation 5*:35-43 (1998); Candinas, D. *et al., Transplantation 62*:1-5 (1996)). Complement inhibitors also include, but are not limited to, antibodies that react against any of the components of the complement pathway, and any drugs or biological agents that inhibit or deplete any components belonging to the complement pathway.

By "blood-organ barrier modifier" is intended an agent that increases the permeability of the blood-organ barrier, which is a system of tight junctions in the capillaries (endothelia) of the organ that resist entry of inflammatory cells, pathogens and macromolecules. Blood-organ barriers include, for example, blood-brain barrier, blood-eye barrier, blood-testis barrier, and blood-nerve barrier. Exemplary blood-organ barrier modifiers include, but are not limited to, bradykinin agonists, such as RMP-7 (for example, Cereport™, Alkermes, Inc., U.S. Patent Nos. 5,506,206 and 5,686,416) and bradykinin, osmotic agents, cyclic GMP modulators, cytokines, nitric oxide modulators, and by physical means.

By "blood-brain barrier modifier" is intended an agent that increases the permeability of the blood-brain barrier, which is a system of tight junctions in the capillaries (endothelia) of the brain that resist entry of inflammatory cells, pathogens and macromolecules. Such blood-brain barrier include the blood-brain-tumor barrier. Exemplary blood-brain barrier modifiers include, but are not limited to, bradykinin agonists, such as RMP-7 (for example, Cereport™, Alkermes, Inc., U.S. Patent Nos. 5,506,206 and 5,686,416) and bradykinin, osmotic agents, cyclic GMP modulators, cytokines, nitric oxide modulators, and by physical means.

By "blood-brain-tumor barrier modifier" is intended an agent that increases the permeability of the blood-brain-tumor barrier, which is a system of tight junctions in the capillaries (endothelia) of the brain tumor that resist entry of inflammatory cells, pathogens and macromolecules. Exemplary blood-brain-tumor barrier modifiers include, but are not limited to, bradykinin agonists, such as RMP-7 (for example, Cereport™, Alkermes, Inc., U.S. Patent Nos. 5,506,206 and 5,686,416) and bradykinin, osmotic agents, cyclic GMP modulators, cytokines, nitric oxide modulators, and by physical means.

Exemplary agents that increase blood volume in brain of a patient include, but are not limited to, ketamine, xylasine, RMP-7, and mannitol. Other blood-organ, blood-brain, or blood-brain-tumor barrier modifiers, mentioned above, can also act to increase blood volume in brain. *See*, LeMay, D.R. *et al., Hum. Gene Ther. 9*:989-995 (1998); Fike, J.R. *et al., J. Neurooncol. 37*:199-215 (1998); Aghi, M. *et al., J. Natl. Cancer Inst. 90*:370-380 (1998).

Exemplary osmotic agents, as mentioned above, for use in various aspects of the invention include, but are not limited to, mannitol and urea. Exemplary cyclic GMP modulators, as mentioned above, for use in the invention include, but are not limited to, dimaprit and zaprinast. Exemplary cytokines, as mentioned above, for use in the invention include, but are not limited to, interleukin-1β, tumor necrosis factor-α and interferon-γ. Exemplary nitric oxide modulators, as mentioned above, for use in the invention include, but are not limited to,

L-arginine, sodium nitroprusside, SIN-T(3-morpholinosydnone imine), diethylamine/NO, diethylenetriamine/NO, spermine/NO, S-nitrous-N-acetyl cysteine, and proline-nitric-oxide. Exemplary physical methods, as mentioned above, for use in the invention include, but are not limited to, radiation and focused ultrasound. A preferred blood-organ, blood-brain, or blood-brain-tumor barrier modifier is RMP-7. *See*, Nilaver, G. *et al., Proc. Natl. Acad. Sci. USA 92*:9829-9833 (1995); Rainov, N.G. *et al., Human Gene Therapy 6*:1543-1552 (1995); Rainov, N.G. *et al., Cancer Gene Ther. 5*:158-162 (1998); Elliott, P.J. *et al., Exptl. Neurol. 141*:214-224 (1996); Bartus, R.T. *et al., Immunopharm. 33*:270-278 (1996); Bartus, R.T. *et al., Exptl. Neurol. 142*:14-28 (1996); LeMay, D.R. *et al., Human Gene Therapy 9*:989-995 (1998).

By "genetic vector" is intended a non-viral or viral vector. By "non-viral vector" is intended a vector that is not derived from a virus. Exemplary non-viral vectors that can be used in the invention include, but are not limited to, prokaryotic vectors (Pawelek, J.M. *et al., Cancer Res. 57*:4537-4544 (1997)), DNA-protein complexes or conjugates, liposomes, direct injection of nucleic acid, receptor-mediated gene transfer, and gene "gun" vector (Agracetus, WI). Other suitable non-viral vectors will be readily apparent to the skilled artisan.

The viral vector of the invention can be derived from several different types of viruses. By "derived from a virus" is meant that the virus is a source of viral DNA for making the viral vector of the invention. Viruses that can be used to derive the viral vectors of the invention include, but not are limited to, herpes viruses, such as herpes simplex virus (e.g., Chase, M. *et al., Nature Biotechnol. 16*:444-448 (1998)), cytomegalovirus, Epstein-Barr virus, varicella zoster virus, psuedorabies virus, retrovirus (e.g., Vile, R.G. *et al., Brit. Med. Bull. 51*:12-30 (1995); U.S. Patent No. 5,763,242), lentivirus, adenovirus (e.g., Brody, S.L. *et al., Ann. N.Y. Acad. Sci. 716*:90-101 (1994); Heise, C. *et al., Nature Med. 3*:639-645 (1997)), adeno-associated virus (e.g., Flotte, T.R. and Carter, B.J., *Gene Ther. 2*:357-362 (1995)), and hybrid virus (e.g., Bilbao, G. *et al., FASEB J. 11*:624-634 (1997)).

Other viruses that can be used to derive the viral vectors of the invention include members of the pox virus family, such as vaccinia virus and smallpox virus, and African swine fever virus.

Herpes simplex viruses are of particular interest. By "herpes simplex virus" is intended any member of the subfamily *herpesviridae alpha* containing a mutation as described above. HSV-1 and HSV-2 are members of this subfamily. HSV-1 is of particular interest.

HSV-1 is a human neurotropic virus that is capable of infecting virtually all vertebrate cells. Natural infections follow either a lytic, replicative cycle or establish latency, usually in peripheral ganglia, where the DNA is maintained indefinitely in an episomal state. HSV-1 contains a double-stranded, linear DNA genome of 153 kilobases in length, which has been completely sequenced (McGeoch *et al., J. Gen. Virol. 69*: 1531 (1988); McGeoch *et al., Nucleic Acids Res 14*: 1727 (1986); McGeoch *et al., J. Mol. Biol. 181*: 1 (1985); Perry and McGeoch, *J. Gen. Virol. 69*: 2831 (1988)). DNA replication and virion assembly occurs in the nucleus of infected cells. Late in infection, concatemeric viral DNA is cleaved into genome length molecules which are packaged into virions. In the CNS, herpes simplex virus spreads transneuronally followed by intraaxonal transport to the nucleus, either retrograde or anterograde, where replication occurs.

The genetic vector will include one or more promoters or enhancers, the selection of which will be known to those skilled in the art. Suitable promoters include, but are not limited to, the SV40 promoter, the retroviral long terminal repeat (LTR), the human cytomegalovirus (CMV) promoter, and others known in the art.

For oncolysis, in a preferred embodiment of the invention, the viral vector contains a mutation in a gene required for replication, whose mammalian homologue is up-regulated by elevated levels of E2F.

Mammalian ribonucleotide reductase (m*RR*) is up-regulated during the G_1 phase of the cell cycle and its transcription is regulated by "free" E2F (DeGregori

et al., *Mol. Cell. Biol. 15*: 4215-4224 (1995); Lukas *et al.*, *Mol. Cell. Biol. 16*: 1047-1057 (1996); Dynlacht *et al.*, *Genes Dev. 8*: 1772-1786 (1994)). It has been hypothesized that RR$^-$ viral mutants selectively replicate in neoplastic cells owing to the presence of the complementing mammalian ribonucleotide reductase (mRR) in these cells (Goldstein and Weller, *J. Virol. 62*: 196-205 (1988)).

Elevation in the levels of free E2F causes increased expression of several mammalian genes whose viral homologues are required for replication of the virus. In addition to ribonucleotide reductase (rr), these genes include thymidine kinase (tk), uracyl-n-glycosylase (ung), and uracyl-triphosphatase enzymes (dUTPase). Viruses containing a mutation in one or more of these genes would replicate selectively in cells with elevated levels of free E2F. Thus, the invention encompasses viral vectors having a mutation in one or more of these genes.

E2F (including E2F1, E2F2, E2F3, E2F4, E2F5) appears to be the primary mediator of the cell cycle-regulated transcriptional cascade that involves p16, cyclin D/cdk4, and pRB (DeGregori *et al.*, *Mol. Cell. Biol. 15*: 4215-4224 (1995); Lukas *et al.*, *Mol. Cell. Biol. 16*: 1047-1057 (1996); Dynlacht *et al.*, *Genes Dev. 8*: 1772-1786 (1994)). Thus, defects in a gene involved in this cascade can lead to increased levels of E2F and thereby increased levels of mammalian RR, TK, UNG and dUTPase. For example, cells with defects in the expression of p16, p21 and/or p27 can have increased levels of cyclin D, cyclin D kinase 4 (Cdk4) and/or cyclin D kinase 6 (Cdk6) which can in turn lead to increased phosphorylation of pRB thereby liberating E2F. In addition, cells with defects in the expression of pRB, p107 and or p130, DP1, DP2, and/or DP3 can also lead to increased liberation of E2F.

The majority of tumors possess an inactivation of a gene encoding a component of this cascade (Ueki *et al.*, *Cancer Res. 56*: 150-153 (1996)), thus liberating E2F and allowing for transcription of mammalian rr, tk, ung, and dUTPase. Moreover, alterations in other tumor suppressor genes or oncogenes can also lead to increased levels of free E2F, and thereby increased levels of mammalian RR, TK, UNG and dUTPase. Therefore, RR$^-$, TK$^-$, UNG$^-$ and

dUTPase viral mutants can effectively target a large percentage of tumor cells, particularly if they possess a defect in the *p16*/cyclin D/p*RB* pathway that leads to an increase in "free" E2F and increased transcription of m*RR*.

Furthermore, a large number of tumor cells from different origins (*e.g.*, lung, breast, prostate, brain, liver, pancrease, skin, etc.) possess alterations in the pathways described above leading to elevated levels of RR, TK, UNG and dUTPase, and thus are targets for the viral mutant of the invention. For example, the tumor cell lines such as rat 9L, human U87, and human T98 cells possess inactivating mutations of *p16* (Van Meir *et al., Cancer Res. 54*: 649-652 (1994)), as well as elevated levels of m*RR*. These cells were thus able to complement the replication of the herpes simplex-1 derived viral mutant rRp450 to levels close to that of the wild-type KOS strain, while neurons with no detectable level of m*RR* (and with a normal *p16* pathway) did not.

A preferred embodiment of the invention is a HSV mutant, hrR3, which has a disruption of the RR gene through insertion of the *Escherichia coli* lacZ gene under the control of the ICP6 promoter (Goldstein, D.J. and Weller, S.K., *J. Virol. 62*:196-205 (1988)).

Ribonucleotide reductase (RR) is a key enzyme in the de novo synthesis of DNA precursors, catalyzing the reduction of ribonucleotides to deoxyribonucleotides. HSV-1 encodes its own RR (UL39 and UL40 genes), which is composed of two non-identical subunits (Duita, *J. Gen. Virol. 64*: 513 (1983)). The large subunit (140K molecular weight), designated ICP6, is tightly associated with the small subunit (38K molecular weight). Herpes simplex virus RR has been found to be required for efficient viral growth in non-dividing cells but not in many dividing cells (Goldstein and Weller, *J. Virol. 62*: 196 (1988); Goldstein and Weller, *Virol. 166*: 41 (1988); Jacobson *et al., Virol. 173*: 276 (1989)). Mutations in the small subunit of RR also lead to loss of RR activity and neuropathogenicity (Cameron *et al., J. Gen. Virol. 69*: 2607 (1988)), however, particularly preferred are mutations in the large subunit.

The promoter region of ribonucleotide reductase ICP6 has been mapped. The promoter region of ICP6 has been mapped to the 5' upstream sequences of the ICP6 structural gene (Goldstein and Weller, *J. Virol. 62*: 196 (1988); Sze and Herman, *Virus Res. 26*: 141 (1992)). The transcription start site for the small subunit of RR, namely UL40, falls within the coding region of ICP6 (McLauchlan and Clements, *J. Gen. Virol. 64*: 997 (1983); McGeoch *et al., J. Gen. Virol. 69*: 1531 (1988)).

Viral vectors derived from HSV-2 based on the viral mutants illustrated herein using the HSV-1 genome are encompassed by the present invention. HSV-2 contains both RR subunits; moreover, HSV-1 ICP6 is analogous to HSV-2 ICP10 (Nikas *et al., Proteins 1*: 376 (1986); McLaughlan and Clements, *EMBO J. 2*: 1953 (1983); Swain and Halloway, *J. Virol. 57*: 802 (1986)).

The selectivity of hrR3 for tumors in the brain is based on a lacZ insertional mutation of the ICP6 viral gene encoding for its ribonucleotide reductase function, necessary for the upregulation of nucleotide pools in infected post-mitotic cells. Lack of this function restricts viral DNA synthesis and replication with subsequent cyto-toxicity and -lysis to cells (such as tumor cells) with elevated nucleotide pools, usually an effect of upregulated mammalian ribonucleotide reductase (mRR) (Goldstein, D. J. and Weller, S. K., *J. Virol. 62*:196-205 (1988); Goldstein, D. J. and Weller, S. K., *Virology 166*:41-51 (1988); Goldstein, D. J. and Weller, S. K., *J Virol 62*:2970-2977 (1988)). In fact, an association between the replication of ICP6-defective herpes simplex viral mutants and presence of elevated levels of mRR in tumor cells has been shown (Chase, M., *et al., Nature Biotech. 16*:444-448 (1998)).

In another embodiment, the invention can also be utilized to deliver a transgene to a target cell for treatment of inborn errors of metabolism, such as Tay-Sachs disease, neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, stroke, spinal cord trauma, and nerve trauma. The genetic vector can contain a transgene capable of correcting the defect that causes the disease.

By "transgene" is intended a heterologous gene that is introduced to the target cell. A transgene is non-native or foreign gene to the target cell.

For example, gene therapy can be used to treat the following diseases: inborn errors of metabolism (lysosomal storage diseases) (Kaye, E.M., GENE THERAPY FOR LYSOSOMAL STORAGE DISEASES in: *Gene Therapy for Neurological Disorders and Brain Tumors*, Chapter 19, Chiocca, E.A. and X.O. Breakfield, ed. (Human Press Inc., Totowa, NJ (1998), Lacorazza *et al., Nature Med. 2*:424-429 (1996)); peripheral nerve injury (Jorensen, J. *et al., Ann. Neurol. 43*:205-211 (1998), and Xu, X.M. *et al., Exptl. Neurol. 134*:261-272 (1995)); motor neuron disease (Gravel, C. *et al., Nature Med. 3*:765 (1997), Sendtner, M., *Nature Med. 3*:380 (1997), and Haase, G. *et al., Nature Med. 3*:429 (1997)); amyotrophic lateral sclerosis (Aebischer, P. *et al., Nature Med. 2*:696 (1996)); Alzheimer's disease (Buxbaum, J.D. *et al., Nature Med. 4*:1177 (1998), and Vito *et al., Science 271*:521-525 (1996)); stroke (Pechan, P.A. *et al.*, GENE THERAPY FOR ISCHEMIC STROKE in: *Gene Therapy for Neurological Disorders and Brain Tumors*, Chapter 18, Chiocca, E.A. and X.O. Breakfield, ed. (Human Press Inc., Totowa, NJ (1998), and Linnik *et al., Stroke 26*:1670-1674 (1995)); Parkinson's disease (Choi-Lundberg *et al., Science 275*:838-841 (1997); as well as other diseases.

In another embodiment, the invention can be utilized to deliver a transgene whose expression product activates a chemotherapeutic agent.

By "selectively killing neoplastic cells" is meant that the viral vector of the invention primarily targets neoplastic cells, rather than non-neoplastic cells. This targeting is due to having a mutation in a viral gene, wherein the viral gene is complemented by its mammalian homologue in mammalian cells in which levels of free E2F are elevated. *See*, Singhal, S. and Kaiser, L.R., *Cancer Gene Therapy 7*:505-536 (1998).

In one example of viral delivery of a suicide gene (a gene with a drug-conditional "killing" function), expression of the HSV thymidine kinase (TK) gene in proliferating cells, was found to render cells sensitive to the deoxynucleoside

analog, ganciclovir (GCV) (Moolten *et al.*, *Cancer Res. 46*:5276-5281 (1986); Moolten *et al.*, *Hum. Gene Ther. 1*:125-134 (1990); Moolten *et al.*, *J. Natl. Cancer Inst. 82*:297-300 (1990)). HSV-TK mediates the phosphorylation of GCV, which is incorporated into DNA strands during DNA replication (S-phase) in the cell cycle, leading to chain termination and cell death (Elion, G. B., *J. Antimicr. Chemother. 12, sup. B*:9-17 (1983)).

Another example of a suicide gene suitable for viral delivery is the cytochrome P450 gene, which confers chemosensitivity to the class of oxazosphorine drugs. Two of these drugs, cyclophosphamide (CPA) and its isomeric analog ifosfamide (IFA) are mainstays of cancer chemotherapy for several types of tumors (Colvin, O. M., in *Cancer Medicine*, Holland *et al.*, eds., Lea and Febiger, Philadelphia, Pa. (1993), pages 733-734). These therapeutically inactive prodrugs require bioactivation by liver-specific enzymes of the cytochrome P450 family. One of these enzymes, cytochrome P450 2B1 ("*CYP2B1*"), which is induced by phenobarbital, activates CPA and IFA with high efficiency (Clarke *et al.*, *Cancer Res. 49*:2344-2350 (1989); Weber and Waxman, *Biochem. Pharm. 45*:1685-1694 (1993)). CPA and IFA are hydroxylated by cytochrome P450 to yield the primary metabolites 4-hydroxycyclophosphamide or 4-hydroxyifosphamide, respectively. These primary metabolites are unstable and spontaneously decompose into cytotoxic compounds: acrolein and phosphoramide (or ifosphoramide) mustard (Colvin *et al.*, *Cancer Treat. Rep. 65*:89-95 (1981); Sladek, in *Metabolism and Action of Anticancer Drugs*, Powis *et al.*, eds., Taylor and Francis, New York (1987), pages 48-90). The latter causes interstrand cross-links in DNA regardless of cell-cycle phase. Maximum cytotoxicity is obtained during subsequent S and mitotic (M) phases of the cell cycle due to strand breaks (Colvin (1993), *supra*). U.S. Patent No. 5,688,773, to Chiocca *et al.* (November 18, 1997), describes a gene therapy paradigm using cytochrome P450 and CPA.

Replication-defective vectors based on retrovirus (Wei *et al.*, *Human Gene Therapy 5*: 969-978 (1994); Chiocca *et al.*, U.S. Patent No. 5,688,773) or

adenovirus (Chen *et al.*, *Cancer Res. 56*: 1331-1340 (1996)) have been employed to achieve transfer into tumor cells of the transgene encoding for rat *CYP2B1*. When treated with CPA, tumor cells that have been engineered to express cytochrome *CYP2B1* generate freely diffusible active CPA metabolites that are cytotoxic to surrounding tumor cells, which may not contain the *CYP2B1* transgene (Chen and Waxman, *Cancer Res. 55*: 581-589 (1995); Wei *et al.*, *Clin. Cancer Res. 1*: 1171-1177 (1995)). Thus, the CPA/cytochrome P450 gene therapy approach can provide a means for intratumoral generation of alkylating metabolite. A viral vector is described in Chase, M. *et al., Nature Biotech. 16*:444-448 (1998), that can both target neoplastic cells for viral mediated oncolysis and deliver a transgene capable of activating or enhancing a chemotherapeutic agent locally, wherein the transgene/chemotherapeutic agent combination does not significantly inhibit viral replication.

By "mutation" is intended any alteration to a gene wherein the expression of that gene is decreased, or wherein the gene product is rendered nonfunctional, or its ability to function is decreased. As used herein, the term "gene" encompasses both the regions coding the gene product as well as regulatory regions for that gene, such as a promoter or enhancer. Such alterations render the product of the gene non-functional or reduce the expression of the gene such that the viral mutant has the properties of the instant invention.

In the invention, by "a" is intended one or more. For example, "a mutation in a ribonucleotide reductase gene" means that there can be one or more mutations in one or more ribonucleotide reductase genes.

Ways to achieve such alterations include (a) any method to disrupt the expression of the product of the gene or (b) any method to render the expressed ribonucleotide reductase nonfunctional. Numerous methods known to disrupt the expression of a gene are known, including the alterations of the coding region of the gene, or its promoter sequence in the by insertions, deletions and/or base changes (*see,* Roizman and Jenkins, *Science 229*: 1208 (1985)).

A preferred mutation is the deletion of nucleic acids from a gene. A more preferred mutation is one wherein the mutation is produced by replacing a significant portion of a gene, by a gene encoding a gene product capable of converting a chemotherapeutic agent to its cytotoxic form, wherein the chemotherapeutic agent does not significantly inhibit replication of the viral mutant.

Methods for the construction of engineered viruses are known in the art. Additional methods for the genetic manipulation of DNA sequences are known in the art. Generally, these include Ausubel *et al.*, Chapter 16 in *Current Protocols in Molecular Biology* (John Wiley and Sons, Inc.); Paoletti *et al.*, U.S. Pat. No. 4,603,112 (July 1986). Virological considerations also are reviewed in Coen, in *Virology,* 1990 (2nd ed.) Raven Press, pages 123-150.

The construction of HSV-1 vectors is described, for example, in Martuza *et al.*, U.S. Pat. No. 5585096 (Dec. 1996); Roizmann *et al.*, U.S. Pat. No. 5,288,641 (Feb. 1994); Roizman and Jenkins, *Science 229*:1208 (1985); Johnson *et al., J. Virol. 66*:2952 (1992); Gage *et al., J. Virol. 66*:5509 (1992); Spaete and Frenkel, *Cell 30*:295 (1982); Goldstein and Weller, *J. Virol. 62*:196 (1988), Coen, chapter 7, in *Virology*, 1990 (2nd ed.) Raven Press; Breakefield and DeLuca, *The New Biologist, 3*:203 (1991); Leib and Olivo, *BioEssays 15*:547 (1993); Glorioso *et al., Seminars in Virology 3*:265 (1992); Chou and Roizman, *Proc. Natl. Acad. Sci. USA, 89*:3266 (1992); Breakfield *et al., Molec. Neurobiol. 1*:339 (1987); Shih *et al.*, in *Vaccines 85*, 1985, Cold Spring Harbor Press, pages177-180; Palella *et al., Molec. Cell. Biol. 8*:457 (1988); Matz *et al., J. Gen. Virol. 64*:2261 (1983); Mocarski *et al., Cell 22*:243 (1980); and Coen *et al., Science 234*:53 (1986).

Genetic alterations can be determined by standard methods such as Southern blot hybridization of restriction endonuclease digested viral DNA, sequencing of mutated regions of viral DNA, new restriction endonuclease sites, enzymatic assay for ribonucleotide reductase activity (Huszar and Bacchetti, *J. Virol. 37*:580 (1981)), Western blot or ELISA analysis of infected cell proteins

with antibodies to RR, and/or lack of replication in or mouse cells for RR- (Jacobson *et al., Virology 173*:276 (1989)). A viral vector that has been mutated in one or more ribonucleotide reductase genes can be isolated after mutagenesis or constructed via recombination between the viral genome and genetically-engineered sequences.

The genetic vectors of the invention can carry an altered gene and/or a transgene that encodes a gene product capable of correcting the defect which causes a disease or capable of activating a chemotherapeutic agent to its cytotoxic form, wherein the activated chemotherapeutic agent does not significantly inhibit viral replication. This transgene can be inserted at any location in the viral genome where the transgene will be expressed, and where the insertion does not affect the ability of the virus to replicate in dividing cells. A preferred location for the transgene is in a gene required for viral replication, whose mammalian homologue is up-regulated by elevated levels of E2F, especially a ribonucleotide reductase gene. Even more preferred, is insertion of the transgene into a ribonucleotide reductase gene containing a mutation.

By "gene product capable of converting a chemotherapeutic agent to its cytotoxic form" is meant a gene product that acts upon the chemotherapeutic agent to render it more cytotoxic than it was before the gene product acted upon it. Other proteins or factors can be required, in addition to this gene product, in order to convert the chemotherapeutic agent to its most cytotoxic form.

By "gene encoding a gene product capable of converting a chemotherapeutic agent to its cytotoxic form" is meant a nucleic acid that upon expression provides a product that can act upon a chemotherapeutic agent rendering it toxic to the cells it contacts. The chemotherapeutic agents for use in the method of the invention do not significantly inhibit replication of the viral mutant. In order for the potentiation of viral vector mediated oncolysis by expression of the gene carried by the viral vector and application of a chemotherapeutic agent to occur, the gene product, chemotherapeutic agent, and

active metabolites of the chemotherapeutic agent must not significantly inhibit replication of the viral vector.

"Gene product" broadly refers to proteins encoded by the particular gene.

By "chemotherapeutic agent" is meant an agent that can be used in the treatment of neoplasms, and that is capable of being activated from a prodrug to a cytotoxic form.

By "cytotoxic" is intended causing or leading to cell death.

In one embodiment, the gene encoding a gene product capable of converting a chemotherapeutic agent to its cytotoxic form, wherein the active chemotherapeutic agent does not significantly inhibit viral replication, is a cytochrome P450 gene. The term "gene encoding cytochrome P450" shall mean a mammalian cytochrome P450 gene such as, P450 2B1, P450 2B6, P450 2A6, P450 2C6, P450 2C8, P450 2C9, P450 2C 11, or P450 3A4. Each of these genes has been linked to activation of the anticancer drugs cyclophosphamide or ifosfamide (Clarke *et al., Cancer Res. 49*:2344-2350 (1989); Chang *et al., Cancer Res. 53*:5629-5637 (1993); Weber and Waxman, *Biochemical Pharmacology 45*:1685-1694 (1993)), and the cDNA sequences of these genes have also been published (Nelson *et al., DNA and Cell Biology 12*:1-51 (1993) and references cited therein; Yamano *et al., Biochem. 29*:1322-1329 (1990); Yamano *et al., Biochem. 28*:7340-7348 (1989)). Moreover, cytochrome P450 can also activate N-methyl cyclophosphamide (N-methyl CPA), methylchloropropylnitrosourea (MCPNU), and polymeric forms of CPA, ifosfamide, N-methyl CPA, and MCPNU. Polymeric forms of chemotherapeutic agents are discussed in Brem, *Biomaterials, 11:* 699-701 (1990); Buahin and Brem, *J. Neurooncol 26:* 103-110 (1995); Tamargo *et al., Cancer Res. 53:* 329-333 (1993); and Langer, *Ann. Biomed. Eng. 23:* 101-111 (1995). Persons of ordinary skill in the art should be able to utilize the method of the present invention with numerous other anticancer drugs that are activated by members of the cytochrome P450 family of enzymes (LeBlanc and Waxman, *Drug Metab. Rev. 20*:395-439 (1989)), as well as with drug-metabolizing cytochrome P450 genes from other species (*e.g.*, mouse, rabbit,

hamster, dog, etc.) that are homologous to cytochromes P450 2B1, P450 2B6, P450 2A6, P450 2C6, P450 2C8, P450 2C9, P450 2C 11, or P450 3A4, and whose cDNA sequences are known (Nelson *et al., DNA and Cell Biology 12*:1-51 (1993)). In a particularly preferred embodiment, cytochrome P450 2BI is used.

By "does not significantly inhibit replication of the viral mutant" is meant that viral replication can occur at a level sufficient to lead to death of the infected cell and to propagate the spread of the virus to other cells.

The use of a chemotherapeutic agent/transgene combination in which the chemotherapeutic agent, or its active metabolites, act instead by crosslinking DNA or by inhibiting DNA repair would not significantly inhibit replication of the viral vector. Thus, such chemotherapeutic agent/transgene combinations are encompassed by the viral mutant and methods of the present invention. A preferred chemotherapeutic agent/transgene combination is cytochrome P450 combined with CPA, ifosfamide, -methyl cyclophosphamide, methylchloropropylnitrosourea or polymeric forms of CPA, ifosfamide, -methyl cyclophosphamide and methylchloropropylnirosourea. A more preferred chemotherapeutic agent/transgene combination is CPA/cytochrome P450 2B1. Other chemotherapeutic agent/transgene combinations for use in the present invention include: CB1954/*E. coli* nitroreductase (Friedlos *et al., Gene Ther. 5:* 105-112 (1998); Green *et al., Cancer Gene Ther. 4:* 229-238 (1997)); topoisomerase I or II inhibitors/enzyme with esterase-like activity, such as, *e.g.*, CPT-11/carboxylesterase (Jansen *et al., Int. J. Cancer 70:* 335-340 (1997); Danks *et al., Cancer Res. 58:* 20-22 (1998)); 4-ipomeanol/cytochrome P450 4B1 (Verschoyle *et al., Toxicol. Appl. Pharmacol. 123*: 193-198 (1993)); and 2-aminoanthracene/cytochrome P450 4B1 (Smith *et al., Biochem. Pharmacol. 50:* 1567-1575 (1995)).

Another advantage of using chemotherapeutic agents whose mechanism of action is the cross-linking of DNA or inhibition of DNA repair enzymes is that these agents are effective against even cells in G_0. Thus, for these agents to be effective in killing neoplastic cells, the targeted cells do not have to be actively

dividing at the time that the drug is administered. This is a significant benefit for tumors in which a large percentage of cells are in G_0.

By "patient" is intended a non-human animal or human. Exemplary candidates for treatment according to the present invention include, but are not limited to, (i) patients suffering from neoplasms, (ii) patients suffering from nervous system tumors (central and peripheral) and (iii) patients having malignant brain tumor, including astrocytoma, oligodendroglioma, meningioma, neurofibroma, glioblastoma, ependymoma, Schwannoma, neurofibrosarcoma, and medulloblastoma, (iv) patients having inborn errors of metabolism, such as Tay-Sachs disease, (v) patients having neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease, and (vi) patients suffering from stroke.

The pharmaceutical compositions of the present invention would be advantageously administered in the form of injectable compositions. A typical composition for such purpose would comprise a pharmaceutically acceptable vehicle. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like (*see Remington's Pharmaceutical Sciences* (18th ed.) Mack Publishing Co. (1990)). Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to routine skills in the art (Goodman and Gilman, *The Pharmacological Basis for Therapeutics* (9th ed.) Pergamon Press (1996)).

Typically, the genetic vector, agent that increases blood volume in brain of a patient, blood-organ barrier modifier, blood-brain barrier modifier, blood-brain-tumor barrier modifier, and/or complement inhibitor would be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for

solution in, or suspension in, liquid prior to injection can also be prepared. The preparation also can be emulsified. The active immunosuppressing agent is often mixed with an excipient which is pharmaceutically-acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the preparation can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH-buffering agents, adjuvants or immunopotentiators which enhance the effectiveness of the genetic vector, agent that increases blood volume in brain of a patient, blood-organ barrier modifier, blood-brain barrier modifier, blood-brain-tumor barrier modifier, and/or complement inhibitor.

The quantity of the genetic vector, agent that increases blood volume in brain of a patient, blood-organ barrier modifier, blood-brain barrier modifier, blood-brain-tumor barrier modifier, and/or complement inhibitor to be administered, both according to number of treatments and amount, depends on the subject to be treated, clinical status, age, weight, capacity of the subject's immune system to synthesize antibodies, and degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual.

Dosages of a particular chemotherapeutic agent can be administered according to current standard clinical practice (*see*, Hubbard, S.M. and Jenkins, J.F., *Chemotherapy Administration: Practical Guidelines* in: CANCER CHEMOTHERAPY: PRINCIPLES AND PRACTICE, Chabner and Collins, eds.(J.B. Lippincott Co., Philadelphia, PA (1990)). For example, standard clinical dosages for CPA in adults range from approximately 600-1000 mg/m^2 (Struck, R.F. *et al.*, *Cancer Research 47*:2723-2726 (1987)). Factors such as clinical status, age and weight of the patient can also contribute to dosage adjustment.

Generally, the treatment will be initiated by intravascular, intracarotid or intravenous administration of the viral vector, agent that increases blood volume in brain of a patient, blood-organ barrier modifier, blood-brain barrier modifier, blood-brain-tumor barrier modifier, and/or complement inhibitor. For the most

part, the genetic vector is provided in a therapeutically effective amount to infect and kill target cells or to sufficiently produce a gene product to correct the defect caused by the disease.

The following Examples serve only to illustrate the invention, and are not to be construed as in any way limiting on the invention.

Examples

Example 1

It was determined whether intravascular administration of hrR3 would lead to transduction of an intracerebral human glioma xenograft established in the brain of an athymic rat. Table 1 shows that administration of hrR3 alone did not transduce tumors in tested animals. Co-administration of the bradykinin agonist RMP-7, which has been reported to increase permeability and to disrupt the blood-brain barrier in tumor tissue (Elliott, P. J., *et al., Ex Neurol. 141*:214-224 (1996); Black, K. L., *et al., J. Neurosurg. 86*:603-609 (1997); Lemay. D. R. *et al., Hum. Gene Ther. 9*:989-995 (1998); Fike, J. R., *et al., J. Neurooncol. 37*:199-215 (1998)) produced a large intratumoral "plaque" (covering approximately 73% of tumor volume) in 1/6 animals. Even in an athymic rat, a non-T cell mediated immune response could have been responsible for the observed inefficiency in transduction. In fact, titers of anti-HSV neutralizing antibodies were shown to rise by day 4 after hrR3 injection and this increase was completely inhibited by treating athymic rats with a single, immunosuppressive dose of cyclophosphamide (CPA) (Fig. 1C). Therefore, when CPA was added to the injection schedule, a dramatic enhancement in the number of transduced tumors was observed (Table 1). These neoplasms now contained numerous LacZ-positive plaques that were also immunopositive for the HSV-encoded antigen thymidine kinase (Figs. 1A and 1B). These results thus indicated that, in an athymic rat, multiple and reproducible transduction events of intracerebral human gliomas by hrR3

could be achieved with the aid of the bradykinin agonist, RMP-7, and the immunosuppressive agent, CPA.

It was determined whether the above treatment produced a therapeutically favorable result. Figure 2 shows that the combination of hrR3, RMP-7 and CPA administered intravascularly achieved a significant ($p<0.001$) increase in survival time, resulting in the long-term survival of a majority of athymic rats harboring a single, intracerebral, human glioma xenograft. Combination of hrR3 with RMP-7 or CPA also produced a minority of long-term survivors consistent with the finding that intratumoral "plaques" were visible in some animals (Table 1). However, none of the other treatments, singly or in combination, resulted in tumor regression, with animals perishing within 26 days of implantation of their brain tumors. Histologic examination of the brain of long-term survivors did not show residual tumor and instead, animal deaths invariably were produced by an enlarging intracerebral tumor mass (data not shown). In two animals that had perished at the 30- and 40-day time point, there was evidence of tumor lysis with adjacent edema in normal cerebrum, possibly indicative of residual neurotoxic effects from hrR3 (data not shown). Analysis of other organs (liver, spleen, lungs, heart) did not show evidence of replicating virus either at the two-day time point or at death (data not shown). These results demonstrated a therapeutically significant effect against an intracerebral tumor after intravascular administration of an oncolytic virus.

Although effective treatment of single brain tumors would in itself represent a significant advance, the most malignant and untreatable forms of intracerebral neoplasms (malignant gliomas and metastatic tumors) manifest as multifocal masses, located throughout the brain. In order to model such illness, human U87dEGFR glioma cells were implanted into three separate locations in the brain of athymic rats. These tumors grew rapidly and reliably, causing animal deaths by day 14 after implantation (Fig. 2B). At day 8 after implantation, animals were treated with CPA and subsequently with intravascular hrR3 and RMP-7. Brains were then harvested two, four and eight days later to analyze the extent of

tumor transduction. Fig. 2A shows that greater than 90% of the tumor cells in all three neoplastic masses within an animal's brain could be positively transduced by this method. Table 2 provides a computer-assisted analysis of percent tumor transduction at the days-2 and -4 point after treatment in experimental animals. These results thus show that multiple tumor foci within an animal brain can be reliably and effectively transduced by intravascular administration of an oncolytic virus.

To determine whether a therapeutically significant effect could be obtained against multiple tumor foci within the brain, animals harboring the three intracerebral tumors were treated. Fig. 2B shows that there is a statistically significant prolongation in the survival of rats harboring three separate intracerebral tumors after treatment with hrR3, RMP-7 and CPA ($P<0.001$). This finding thus provided evidence for the efficacy of this treatment against multiple intracerebral neoplasms.

It has been shown that the efficiency of transduction of a single intracerebral tumor located within an animal's brain after intravascular administration of hrR3 can be significantly improved by administering RMP-7 (and possibly other modulators of tumoral blood volume) together with immunosuppressive medication. Inhibition of humoral and cellular antiviral responses is required to achieve apparent tumor cures even in the case of multiple intracerebral tumors. Clinically, this can be achieved with transient immunosuppression to inhibit antiviral responses during the process of tumor transduction and lysis. If needed, viral replication can also be stopped with acyclovir/ganciclovir and reversal of immunosuppression. Engineering additional anticancer genes within the oncolytic viral backbone should also enhance therapeutic effectiveness (Aghi, M. *et al., J. Natl. Cancer Inst. 90*:370-380 (1998)). The advent of advanced neuroradiologic techniques including superselective catheterization of small intracerebral arteries provides the technical and clinical expertise for treating multiple intracranial tumors with intra-arterial oncolytic virus, RMP-7 and transient immunosuppression.

Presence of a CPA-labile, innate antiviral activity in rat plasma. Intra-arterial administration of virus should result in initial infection of some cells in the tumor with initial generation of progeny viruses (12-18 hours) and subsequent rounds of infection and propagation (24-48 hours). The results of Figure 1C showed that neutralizing antibody responses, detected at the four-day time point, were not likely to affect these early processes. However, pre-incubation of hrR3 with complement-depleted, undiluted plasma, prepared from pre-immune immunocompetent (Fig. 3A) or athymic rats (Fig. 3B) significantly reduced *in vitro* viral transduction. These results indicated that an innate antiviral activity was present in rodent plasma. CPA suppressed the antiviral effect of undiluted, immunocompetent rat plasma by approximately 30% (Fig. 3A) and that of athymic rat plasma (1:2 dilution) by approximately 20% (Fig. 3B). These results indicated that animal exposure to CPA led to a partial suppression of this innate antiviral activity.

To determine if this antiviral activity required initial contact with virus or directly protected cells from viral infection, plasma was added onto Vero cells in culture before assaying for hrR3 activity. In this case, there was no inhibition of viral transduction, indicating that this activity acted directly on virus (Figs. 3C and 3D).

If the activity was not innate but an early elicited response undetected by the serological experiments shown in Figure 1C, pre-exposure of animals to virus for 2 days (i.e. before the detectable rise in neutralizing antibody titers) would be expected to augment plasma's ability to inactivate virus. However, no difference was detected in the antiviral activity of plasma prepared from pre-immune rodents or from rodents exposed to virus for 2 days (Figs. 3E and 3F). There also was no difference in the extent of suppression of this activity by CPA. This indicated that short-term (i.e., before the formation of neutralizing antibodies) viral exposure did not change the magnitude of the innate activity. Taken in conjunction, these results indicated that there was an innate antiviral activity in rodent plasma that was different from complement (Figures 3A, 3B, 3E, and 3F), was partially

suppressed by CPA (Figs. 3A, 3B, 3E, and 3F), and had to interact with virus before protecting cells from infection (Figs. 3C and 3D).

The CPA-labile, innate antiviral activity is present in humans. The above experiments could be explained if a natural antibody against HSV (a human virus) was present in rodent plasma. Plasma from 3 out of 20 human patients, who did not possess pre-existing antibodies to HSV, was collected before and after they underwent chemotherapy with CPA. This plasma, after complement depletion, still possessed antiviral activity, which was suppressed by CPA treatment (Fig. 4A). The innate antiviral action was even stronger, but was still partially suppressed by CPA (Fig. 4B), if complement was not inactivated. This result indicated that humans also possess innate antiviral function(s) that can be suppressed by CPA. To further test the natural antibody hypothesis, hrR3 was grown in rodent cells. This would allow the virus to acquire an envelope from rodent membranes with its antigenic characteristics (Welsh, R.M., *et al., J. Virol.* *72*:4650-4656 (1998)). However, there was no difference in the antiviral activity of rodent plasma against hrR3 grown in rodent, human, or Vero (monkey) cells (data not shown), again suggesting that a natural antibody did not provide a suitable explanation for this activity.

The innate antiviral activity interacts with complement. Although the total plasma level of complement was not affected by CPA (data not shown), we sought to determine if its function was. Serially diluted and unheated ('complement-rich") plasma, from immunocompetent or athymic rats, was more active in inhibiting virus than, complement-depleted, heated plasma (Fig. 3A vs Fig. 5A and Fig. 3B vs Fig. 5B). Fig. 5A shows that immunocompetent rat plasma inactivated more than 99% of the virus at a dilution of 1:16. CPA suppressed this by approximately 10%. Fig. 5B shows that athymic rat plasma inactivated approximately 80% of the virus at a dilution of 1:32. CPA suppressed this by approximately 60%. Cobra venom factor (CVF) inhibits complement by depleting

C3 and later components (Cochrane, C.G., *et al.*, *J. Immunol.* *105*:55-69 (1970)). The antiviral activity of unheated plasma prepared from CVF-treated animals was also inhibited (Figs. 5A and 5B). EGTA-Mg treatment depletes Ca2+ , an ion needed to activate complement through the classical (James, K., *Am. J. Med. Technol.* *48*:735-742 (1982)) or lectin (Childs, R.A., *et al.*, *Biochem. J.* *262*:131-138 (1989)) pathways. EGTA-Mg pretreatment of unheated plasma was also suppressive (Figs. 5A and 5B). These results suggested that the innate antiviral activity interacted with complement in a Ca^{2+}-dependent manner and that CPA's suppression of this activity altered complement's antiviral function.

Further characterization of the innate antiviral activity. Complement activation can occur by interaction with immunoglobulin ("classical pathway") or lectin (lectin pathway) in a Ca^{2+}-dependent manner or spontaneously ("alternative pathway") in a Ca^{2+}-independent manner. The above results provided evidence for the former possibilities. Preliminary experiments appeared to exclude interaction with lectin (Ikeda and Chiocca, data not shown). However, Figure 6A shows that pretreatment of plasma with rabbit IgG raised against rat IgM reversed the innate antiviral activity of plasma, in a dose-dependent manner, while treatment with control rabbit pre-immune IgG did not. This suggested that pre-immune IgM contributed to the innate antiviral activity of plasma. In addition, plasma was prepared from CPA-treated (or control) rodents and then pre-incubated with rabbit anti-rat IgM. Figure 6B shows that the CPA and rat anti-IgM treatments generated an even more prominent reversion of innate antiviral activity. At this dilution of plasma (1:8), there is little or no effect from CPA treatment (see Figure 5B), but antibody neutralization of rat IgM resulted in further increase in virus survival after treatment with CPA (Figure 6B). These results indicate that CPA decreased the concentration of rodent preimmune IgM available to interact with the oncolytic virus (Figure 6B).

Further support for CPA's role in reversing pre-immune IgM's role in the suppression of viral activity was provided by measuring immunoglobulin plasma

levels in rodents before and after treatment with CPA. The average level of IgM in plasma was 50.4 mg/ dl before CPA (n = 4), but had significantly decreased to 19.6 mg/dl, two days after CPA treatment. There was no alteration in complement levels. Taken in conjunction with previous results, these data indicate that pre-immune IgM contributed significantly to the observed innate antiviral activity.

Based on these results, we thus asked if CPA increased the survival of oncolytic virus in intracerebral human glioma xenografts, directly injected with hrR3. Tumors were explanted two days and four days after direct stereotactic injection of hrR3 with or without concomitant administration of CPA in order to measure yields of surviving virus. At two days, there were approximately $1x10^6$ pfus of hrR3 within tumors harvested from animals (n = 3) treated with CPA, while there were only $1x10^5$ pfus of hrR3 within tumors harvested from animals (n = 3) that had not been treated with CPA. By four days, there were still $1x10^6$ pfus of hrR3 within tumors of CPA-treated animals, but only $1x10^2$ pfus in controls. These results thus directly correlated the *in vitro* evidence showing an effect of CPA on increasing virus survival on exposure to plasma with *in vivo* measurements showing an effect of CPA on augmenting the survival of oncolytic virus within injected tumors.

Materials and Methods

HSV-1 vector stocks. The virus used in this study was the genetically engineered HSV mutant, hrR3, derived from HSV-1 KOS, which has an intact TK gene and a disruption of the ribonucleotide reductase (RR) gene through insertion of the Escherichia coli lacZ gene under the control of the ICP6 promoter (Goldstein, D. J. and Weller, S. K., *J. Virol. 62*:196-205 (1988)). Viral stocks were generated in African green monkey kidney cell culture (Vero) and titered by plaque assays as described elsewhere (Roizman, B. and Spear, P. G., *J. Virol. 2*:83-84 (1968)). As a control, a mock-infection extract was prepared from mock-

infected cells using the same procedures. A total of $1X10^9$ pfu aliquots in 100 µl were introduced into the carotid artery as a bolus injection. The virus was also grown in U87dEGFR cells and passaged three times before antiviral activity assay.

Cell culture. U87dEGFR cells were a generous gift of Dr. H.-J. Su Huang (University of California at San Diego). This cell line was established by retroviral transfer of a mutant EGFR receptor (de 2-7 EGFR) into U87 human glioblastoma cell line, enhancing its tumorigenic capacity in brain of nude mice (Nishikawa, R. *et al., Proc. Natl. Acad. Sci. USA 91*:7727-7731 (1994)). Human U87 dEGFR cells were propagated at 37°C in an atmosphere containing 5% CO2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml G418 (Sigma).

Assays for antiviral activity in plasma. In addition to rodent plasma, human plasma was collected from 20 patients before and 2 days after they underwent chemotherapy with CPA. Plasma samples were incubated for 30 min at 56C and then serially diluted with PBS (starting at a 1:2 dilution). EGTA-Mg-treated plasma contained 10mM EGTA and 7mM $MgCl_2$. One hundred µl of plasma was incubated with 2 x 104 pfu of hrR3 for 1.5 hours at 37 C and applied onto 4 x 104 Vero cells in 24-well plates. Sixteen hours later, cells were fixed and stained for β-galactosidase activity. Neutralizing antibody titers were determined by the highest dilution of heated plasma which reduced the number of β-galactosidase positive cells by 50% or more, when compared to the control. Antiviral activity of plasma was shown as percentage of the number of β-galactosidase positive cells, when compared to the control. In pilot experiments, the ability of hrR3 to form a lacZ-positive plaque in Vero cells (4-5 days) was essentially identical to its ability to initially transduce a cell. For antibody neutralization studies, rabbit IgG raised against rat IgM and rabbit pre-immune IgG were purchased from Accurate Chemical & Scientific

Corporation, Westbury, NY. Increasing doses of antibodies were then incubated with 1: 4 diluted unheated nude rat plasma (final plasma dilution 1:8) for 1 hour at room temperature before incubation with virus for 1.5 hour at 37C. The mixture was then used to infect Vero cells to assay for effects on viral activity.

Animal studies. Adult female nude rats (rnu/rnu) were anesthetized with an intraperitoneal injection of 0.5 ml of 0.9% NaCl containing 12.5 mg of ketamine and 2.5 mg of xylazine. After immobilizing the rats in a stereotactic apparatus and placing a linear skin incision over the bregma, a 1 mm burr hole was drilled in the skull approximately 1 mm anterior to and 2 mm lateral to the bregma on the right side. For the multi-focal tumor model, two additional burr holes were drilled. The first was 1 mm anterior to and 2 mm lateral to the bregma on the left side, and the other was 3 mm posterior and 2 mm lateral to the bregma on the right side. Two hundred thousand U87dEGFR cells (in a 2 μl volume) were injected at a depth of 3.5 mm from the dura, using a 5 μl Hamilton syringe.

Eight days later, cyclophosphamide (100 mg/kg) or vehicle was injected intraperitoneally, followed by intra-arterial catheterization. The catheterization technique used was essentially identical with the one previously described by Rainov *et al.* (Rainov, N. G. *et al.*, *Hum. Gene Ther.* *6*:1543-1552 (1995)). RMP-7 at a dose of 1.5 μg/kg or vehicle was infused over 10 min. Midway through the infusion, a 100 μl bolus of virus (10^9 pfu) or vehicle was given. For virus distribution studies, virus ($2x10^7$ PFU in 2 μl) was inoculated stereotactically into 8 day old intracerebral U87dEGFR tumors and then rats were killed 1, 2, 3 or 4 days later. For virus distribution and toxicity assays, animals were sacrificed and then perfused by intracardiac infusion of a solution containing 4% neutral paraformaldehyde in PBS. Their brain tumors were aseptically explanted, weighed and then minced. Tissue was homogenized in HBSS containing collagenase/dispase at 1 mg/ml (Boehringer Mannheim, Boehringer, Germany) and then incubated at 37C for one hour. Virus was then harvested from the tissue by freeze-thawing and ultrasonication and then assayed by plaque assays on Vero

cells. For lacZ-expression and toxicity assays, animals were anaesthetized and them perfused by intracardiac infusion of a solution containing 4 % neutral paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brain, liver, lung, kidney, and heart were removed and placed in 4% PFA-PBS overnight at 4°C. They were then transferred to 30% sucrose-PBS for 2 days, frozen over liquid nitrogen and stored at -80°C. For survival studies, rats were observed twice daily until they exhibited neurologic impairment (inability to feed, drink, or move). For neutralizing antibody assays, blood samples were drawn from the heart and stored at -80°C. Blood samples were also obtained from rats that had been injected intraperitoneally with CPA on day -2 and/or with cobra venom factor (Quidel, San Diego, CA) on day -1 at a dose of 60 U/kg and on day 0 at 20 U/kg (days numbered with respect to the day rats were killed). Survival analysis was performed by the statistical software StatXact using Kaplan-Meier survival estimation and the Wilcoxon signed rank test for significance.

Sectioning and histochemistry for virus distribution assay. Brains were analyzed by sectioning (20 μm thick) on a cryostat and air-dried at room temperature. Sections were stained either by histochemistry using the X-Gal substrate, as previously described (Turner, D. L. *et al., Neuron 4*:833-845 (1990)) or by immunohistochemistry using rabbit polyclonal antibodies against highly purified HSV-TK (a gift from Dr. W. Summers, Yale University School of Medicine) and were counter-stained either in Neutral Red or in Hematoxilin.

Quantitative analysis of virus distribution by a computer-assisted method. Three representative sections surrounding the injection site were selected from each tumor and were analyzed using an Olympus BX60 microscope. The sections were scanned by Sony 3-chip Color Video Camera at 20x magnification and the entire tumor area and β-galactosidase positive area were measured using Image Pro Plus Imaging Software.

Table 1. LacZ gene transduction and viral plaque formation in multiple intracerebral tumors two days after intravascular delivery of oncolytic virus.

CPA	RMP-7	No. of animals with intratumoral "plaques"	% intratumoral LacZ gene transduction[A]
-	-	0/4[B]	
-	+	1/6	73.7 (4.6)[C]
+	-	1/4	2.8 (0.2)
+	+	6/8	10.2 (3.6)
			51.3 (8.9)
			33.9 (9.2)
			2.7 (0.4)
			25.6 (7.5)
			18.8 (6.5)

[A]Percentages were calculated by computer-assisted analysis of lacZ-expressing tumor area divided by total tumor area.

[B]Ratios represent the number of animals with visible, lacZ-expressing intraneoplatic "plaques" divided by the number of treated animals.

[C]Values represent the mean, with s.e.m. in parentheses.

Table 2. LacZ gene transduction and viral plaque formation in multiple intracerebral tumors two (rats No. 1 through 8) and four (rats No. 9 through 13) days after intravascular delivery of oncolytic virus.

	% intratumoral LacZ gene transduction		
Rat No.	**Tumor 1[B]**	**Tumor 2**	**Tumor 3[A]**
1	10.3 (4.4)[C]	2.6 (1.5)	0.6 (0.3)
2	0	0	0
3	0	-[D]	0
4	4.0 (0.5)	0	0
5	0	-[D]	1.0 (0.3)
6	4.0 (0.9)	0	0
7	1.5 (0.7)	0	0
8	0	3.9 (1.9)	0
9	97.7 (1.9)	92.5 (2.5)	92.2 (3.4)
10	0	0	0
11	89.3 (1.1)	82.2 (4.0)	82.1 (7.9)
12	14.9 (2.3)	0	0
13	0	65.1 (20.2)	0

CPA and RMP7 were administered to all rats.

[A]Percentages were calculated by computer-assisted analysis of lacZ-expressing tumor area divided by total tumor area.

[B]Tumor 1, 2, and 3: right frontal, right thalamic and left frontal cerebral neoplasms, respectively.

[C]Values represent the mean, with s.e.m. in parentheses.

[D]Tumor did not establish in this location in this rat.

Discussion

The exploration of host immune interactions with oncolytic virus is not only biologically important, but also relevant for its therapeutic implications. Successful delivery of viruses and vectors through the vasculature may provide a means to potentially target multiple tumors within an organ, a feat that has not been currently achieved in an efficient manner. In this study, we discovered that this inefficiency was related to inactivation of the vector in rodent plasma and tumor. This inactivation could be partially circumvented by a single dose of cyclophosphamide. Addition of this agent significantly increased virus survival within the tumor, thus augmenting the number of animals whose tumors were positively infected and the anatomic extent of tumor transduction. This led to a significant prolongation of animal survival, by involution of the brain tumors. This was achieved in animals that had three separate intracerebral tumors, were treated when their illness was clinically detectable (on day 8 of a 14-day life span from tumor cell implantation), and showed radiologic evidence of intracerebral shift and edema.

Enduring protective, T-cell independent, IgM responses can be generated against some viruses (Fehr, T., *et al.*, *Nature Med. 4*:945-948 (1998)) and our results clearly indicate that such a response is elicited against HSV. The B-cell immunosuppressive agent cyclophosphamide (CPA) abrogated this response, which was detectable 4 days after virus administration. However, this time period fails to explain the significant differences in the extent and number of transduced tumors observed 2 days after intra-arterial viral administration in the presence of CPA. An innate antiviral activity was thus discovered in plasma whose function was partially suppressed by CPA and several experiments were conducted to characterize it. Clues to its identity were provided by experiments showing that: 1) rat preimmune IgM plasma levels decreased by more than half, two days after treatment of animals with CPA (through its effects on B-cell production of immunoglobulin), 2) plasma treatment with anti-IgM removed this innate activity

in a dose-dependent fashion, 3) this innate activity affected complement function, but was not inactivated by the usual treatments used to remove complement, and 4) depletion of calcium suppressed it. We propose that preimmune IgM contributes to this innate activity, by binding to virus and activating complement through the classical pathway. Additional support for complement's involvement is provided by the finding that its depletion with CVF also suppressed the innate antiviral action (see Figure 5B). Other possibilities do not seem as likely, such as cell-mediated immune responses and their products, because they are elicited late after viral administration (Herrlinger, U., *et al.*, *Gene Ther. 5*:809-819 (1998); Wood, M.J.A., *et al., Gene Ther. 1*:283-291 (1994)) and because of the use of athymic animals. The CPA-labile activity had to interact first with virus to protect cells from viral infection, thus suggesting that it was not cytokine-based. Additional possibilities remain the focus of current investigation: they do not seem as likely, because one would have to provide a novel mechanism for CPA-mediated suppression of these molecules. These include a mannose-binding protein which binds foreign antigens and activates complement in a calcium-dependent fashion (lectin pathway (Childs, R.A., *et al.*, *Biochem. J. 262*:131-138 (1989))), and/or serum lipoproteins, that are known to bind and inactivate HSV (Huemer, H.P., *et al.*, *Intervirology 29*:68-76 (1988)). Further evidence against a role for the latter group of molecules in our experimental paradigm is their reported independence of Ca2+ for HSV inactivation (Huemer, H.P., *et al.*, *Intervirology 29*:68-76 (1988)) and lack of known interactions with complement.

These findings are relevant to the use of oncolytic HSV vectors for the treatment of human tumors. Human plasma was found to inactivate viral transducing ability to a magnitude that was similar to that of immunocompetent rodent plasma. CPA partially reversed this activity, suggesting that its administration would improve the efficacy of current trials of oncolytic viral therapy for cancer (Rampling, R., *et al.*, *Nature Med. 4*:133 (1998)). The presence of this innate activity in both human and rodent plasma also suggested

that it was unlikely to represent a natural antibody similar to the anti-Gal natural antibody found in human serum against murine retroviruses (Rother, R.P., *et al.*, *J. Exp. Med. 182*:1345-1355 (1995); Takeuchi, Y., *et al.*, *Nature 379*:85-88 (1996)).

The current evidence favors a model in which intravascular (or intratumoral) injection of oncolytic virus results in viral aggregation by preimmune IgM. Although by itself the strength of this binding is relatively low (since it is present only in plasma at a maximal dilution of 1:2 , see Figures 3A and 3B), these aggregates are likely to activate complement through the calcium-dependent classical pathway, significantly enhancing plasma's ability to inactivate virus, even at dilutions of up to 1 : 32 (see Figure 5). Additionally, large viral aggregates are likely to be inhibited in their ability to infect the cells and/or cross the tight junctions that compose the blood-brain barrier. CPA treatment of animals does not affect complement levels itself, but is known to inhibit production of pre-immune immunoglobulins (IgM and IgG) by B cells. CPA-mediated depression of circulating levels of IgM leads to decreased antiviral complement activity, thus increasing survival of virus within tumors. In fact, in our experiments, survival of virus in CPA-treated animal tumors was found to be higher than that in control tumors by one logarithmic unit at 2 days and by four logarithmic units at 4 days, when the titer of neutralizing antibodies is expected to rise. At this point, CPA's initial permission for virus survival within infected tumors combined with its abrogation of neutralizing antibody formation sets up the intraneoplastic propagation of oncolytic virus' progeny and the involution of tumors, observed at the later stages of our experiment (days 4-8).

Herpes simplex viruses have evolved several mechanisms to escape recognition by the immune system, such as the inactivation of the C3 component of complement by HSV's glycoprotein C (gC) and the inactivation of some humoral antiviral functions by binding of non-immune and immune Fc IgG by glycoproteins E and I (gE and gI) (Nagashunmugam, T., *et al.*, *J. Virol. 72*:5351-5359 (1998); Lubinski, J.M., *et al., J. Virol. 72*:8257-8263 (1998)). Not

much is known related to HSV's ability to inactivate IgM. The findings of this report may be generalized to other HSV strains, because a similar innate activity inactivated a HSV mutant from strain F and a HSV amplicon derived from strain 17, but not a VSV-G pseudotyped retroviral vector.

Oncolytic viruses can provide selective anticancer effects in experimental tumor models (Chase, M., *et al.*, *Nature Biotech. 16*:444-448 (1998); Martuza, R.L., *Science 252*:854-856 (1991); Boviatsis, E.J., *et al., Cancer Res. 54*:5745-5751 (1994); Boviatsis, E.J. *et al.*, *Gene Therapy 1*:323-331 (1994); Mineta, T., *et al.*, *Nature Med. 1*:938-943 (1995); Bischoff, J.R., *et al.*, *Science 274*:373-376 (1996); Kucharczuk, J.C. *et al.*, *Cancer Res. 57*:466-471 (1997); Andreansky, S., *et al.*, *Cancer Res. 57*:1502-1509 (1997); Pyles, R.B., *et al.*, *Hum. Gene Ther. 8*:533-544 (1997); Kramm, C.M., *et al., Hum. Gene Ther. 8*:2057-2068 (1997); Coffey, M.C., *et al., Science 282*:1332-1334 (1998)). Addition of anticancer genes to the viral genome can also augment oncolysis (Chase, M., *et al.*, *Nature Biotech. 16*:444-448 (1998); Boviatsis, E.J., *et al., Cancer Res. 54*:5745-5751 (1994); Martuza, R.L., *Science 252*:854-856 (1991)). In fact, we have generated an ICP6 mutant that expresses the CYP2B1 gene responsible for the activation of the prodrug cyclophosphamide into its active anticancer and immunosuppressive metabolites (Chase, M., *et al.*, *Nature Biotech. 16*:444-448 (1998)). This mutant and other HSV mutants should be safer and more effective than hrR3 for further testing of intravascular administration in appropriate primate models. Ultimately, superselective arterial catheterization techniques provide an avenue for safety and efficacy clinical trials in patients afflicted with refractory, multifocal tumors in an organ.

Example 2

The identification of complementing interactions between viral genes and cellular pathways involved in tumorigenesis is providing a biological justification for the use of replication-conditional (oncolytic, replication-restricted) lytic viruses

as anticancer agents (Bischoff, J.R., *et al., Science 274*:373-376 (1996); Boviatsis, E.J., *et al., Hum. Gene Ther. 5*:183-191 (1994); Chase, M., *et al., Nat. Biotechnol. 16*:444-448 (1998); Coffey, M., *et al., Science 282*:1332-1334 (1998); Goldstein, D.J. & Weller, S.K., *Virol. 166*:41-51 (1988); Lorence, R.M., *et al., Cancer Res. 54*:6017-6021 (1994); Lorence, R.M., *et al., J. Natl. Cancer Inst. 86*:1228-1233 (1994); Martuza, R.L., *et al., Science 252*:854-856 (1991)). Even before a full understanding of such interactions, clinical experimental safety trials of viral mutants based on replicating herpes simplex virus or adenovirus are being conducted for refractory head and neck, ovarian, and malignant glioblastoma (Kirn, D., *et al., Nat. Med. 4*:1341-1342 (1998)). These tumors (and other malignant tumors) commonly are refractory to treatment by chemo- or radio-therapy and herald the rapid demise of the afflicted individual. Further complicating treatment is the finding that several malignant tumors manifest as multiple, discrete masses within an organ. This can be seen commonly with metastatic cancers to the liver, brain, and lung. Furthermore, the most malignant form of primary brain tumor (glioblastoma multiforme) can manifest as multicentrically distinct masses within the brain, a universally fatal occurrence (Barnard, R. O. & Geddes, J.R., *Cancer 60*:1519-1531 (1987); Silbergeld, D.L., *et al., J. Neurooncol. 10*:179-185 (1991); Wallner, K.E., *et al., Int. J. Radiat. Oncol. Biol. Phys. 16*:1405-1409 (1989)).

Clinical trials of adeno- or HSV viruses primarily employ direct inoculation into the neoplastic mass through free-hand, stereotactic, or catheter-based techniques (Alavi, J.B., *et al.*, "Phase I trial of gene therapy in primary brain tumors" (Abstract), in *American Soc. Gene Ther. -- First Ann. Meeting (Programs & Abstracts)*, Seattle, WA (1998), p. 444; Berger, M.S., *et al., J. Neurosurgery*:378A (1997); Ram, Z., *et al., Nat. Med. 3*:1354-1361 (1997); Roth, J.A., *et al., Semin. Oncol. 25*:33-37 (1998); Trask, T.W., *et al.*, "A phase I study of adenoviral vector delivery of the HSV-TK gene and the intravenous administration of ganciclovir in adults with malignant tumors of the central nervous system" (Abstract), *Amer. Soc. Gene Ther. -- First Ann. Meeting*

(Programs and Abstracts), Seattle, WA (1998), p. 445). This route of vector administration, while likely to produce focal necrosis within the injected tumor mass, is unlikely to directly generate viral lytic effects against other tumors located within the same organ. Immune-based "cross-priming" reactions may aid in this respect (Toda, M., *et al., Hum. Gene Ther. 10*:385-393 (1999)), but clearly the efficacy of any anticancer action would be greatly improved if some of the injected viruses were able to infect and replicate selectively within each of the tumor masses located in an organ. Therefore, the advantage of injecting oncolytic viruses within the circulation is related to the potential delivery into different tumor masses, commonly supplied by exuberant neovascular growth, with the possibility for lytic action against each tumor focus. However, several potential disadvantages of this approach may be envisioned: 1) It is likely that very few viral particles would be distributed into the neoplasms and infect its cells from the vasculature, 2) Toxic side-effects may occur to normal cells within organs affected by the neoplasm and/or to other organs, 3) Injected viral particles may be inactivated by blood components, and 4) When trying to deliver molecules or viruses from the circulation into the brain, an additional limitation is presented by the blood-brain-barrier (BBB) and the blood-brain tumor-barrier (BTB) (Bartus, R.T., *et al., Exp. Neurol. 142*:14-28 (1996); Elliott, P.J., *et al., Exp. Neurol. 141*:214-224 (1996); Emerich, D.F., *et al., Br. J. Cancer 80*:964-970 (1999); Kroll, R.A. & Neuwelt, E.A., *Neurosurg. 42*:1083-1099, discussion 1099-1100 (1998); Nilaver, G., *et al., Proc. Natl. Acad. Sci. USA 92*:9829-9833 (1995)).

In an attempt to circumvent such limitations, regional delivery of viral vectors through the arterial blood supply may improve the chances of tumor infection, by eliminating "first-pass" effects by the liver. In fact, in experimental models of metastatic liver cancer, delivery through the portal supply to the liver can lead to tumor infection by replication-conditional HSV (Yoon, S., *et al.*, "Treatment of multiple liver metastasis by intravascular delivery of an oncolytic HSV" [submitted to *FASEB J.*]) or by replication-defective adenovirus vectors (Anderson, S.C., *et al., Clin. Cancer Res. 4*:1649-1659 (1998)). However, when

applied to the brain through intracarotid inoculation, no infection of tumors was observed (Rainov, N.G., *et al., Hum. Gene Ther. 6*:1543-1552 (1995)). In an effort to improve this result, the addition of disruptors of the BBB, such as mannitol (Nilaver, G., *et al., Proc. Natl. Acad. Sci. USA 92*:9829-9833 (1995)), or of disruptors of the BTB, such as bradykinin (Rainov, N.G., *et al., Hum. Gene Ther. 6*:1543-1552 (1995)) or its agonist RMP7 (Barnett, F.H., *et al., Cancer Gene Ther. 6*:14-20 (1999)), was shown to facilitate infection of brain tumors by intra-arterial HSV or adenovirus. In spite of this result, the efficiency of tumor infection in the brain, as measured by the number of animals with positively infected tumors and by the anatomic extent of viral infection and/or propagation within a tumor, remained relatively poor (Ikeda, K., *et al., Nature Med. 5*:881-887 (1999)). Concern about this perceived inefficiency thus has led us to consider other physiologic and/or molecular mechanisms that may contribute to this apparent inefficiency.

The inactivation of virus by blood components provides a likely mechanism that may limit the efficacy of intracerebral tumor infection. Serum lipoprotein (Heumer, H.P., *et al., Intervirol. 29*:68-76 (1988); Srinivas, R.V., *et al., Virol. 176*:48-57 (1990); Srinivas, R.V., *et al., J. Cell. Biochem. 45*:224-237 (1991)), fatty acids (Ash, R.J., *Virol. 155*:584-592 (1986); Thormar, H., *et al., Antimicrob. Agents Chemother. 31*:27-31 (1987)), immunoglobulin (Costa, J., *et al., Nature 269*:251-252 (1977); Johansson, P.J. & Kjellen, L., *Intervirol. 29*:334-338 (1988)), and complement (Dubin, G., *et al., Curr. Top. Microbial. Immunol. 179*:111-120 (1992); Lubinski, J.M., *et al., J. Virol. 62*:8257-8263 (1998); Smiley, M.L. & Friedman, H.M., *J. Virol. 55*:857-861 (1985)) have been reported to bind to HSV and inactivate its ability to infect cells. Immunoglobulin has also been reported to bind to infected cells and inhibit further HSV infection by immune-mediated lysis through antibody-dependent cellular cytotoxicity (Kohl, S., *Rev. Infect. Dis. 13*:108-114 (1991)), opsonization of viral particles and/or cells by macrophages (Kodukula, P., *et al., J. Immunol. 162*:2895-2905 (1999)), and activation of classical complement pathways (Kodukula, P., *et al., J.*

Immunol. 162:2895-2905 (1999)). To escape such innate antiviral responses, HSV has been shown to employ several mechanisms. First, the viral glycoprotein gC can bind to and inactivate the C3 component of complement (Lubinski, J., *et al., Semin. Cell. Dev. Biol. 9*:329-337 (1998); Lubinski, J.M., *et al., J. Virol. 62*:8257-8263 (1998)). Second, the viral glycoproteins gE and gI have been shown to bind to the Fc portion of IgG and thus inhibit its function, possibly by antibody bipolar bridging (Dubin, G., *et al., J. Virol. 68*:2478-2485 (1994)). Further evasion of immune responses derives from the function of the viral immediate-early gene transcript, ICP47, that inhibits TAP activity and MHC1 class presentation in infected cells (Goldsmith, K., *et al., J. Exp. Med. 187*:341-348 (1998)). The interaction between the innate immune response against virus and the viral evading mechanisms must represent an important variable in governing the efficacy and toxicity of lytic virus-mediated destruction of tumors, particularly on exposure of the viral vector to host blood components.

To further investigate such aspects, we have recently showed that pre-immune plasma harvested from athymic and immunocompetent rodents as well as from humans can inactivate the *in vitro* transducing ability of the replication-conditional HSV mutant, hrR3 (Ikeda, K., *et al., Nature Med. 5*:881-887 (1999)). This innate activity is present at dilutions as high as 1:32 for athymic and 1:16 for immunocompetent rodents, it is calcium-dependent, it is partially suppressed by in vivo pre-treatment of rodents with agents that deplete complement, such as cobra venom factor (CVF), and it is partially lost upon mild heat inactivation, indicating that one of its components is complement. Additional insights into the characterization of this activity were provided by antibody neutralization studies showing that pre-immune rodent IgM was also contributory to this innate activity and that *in vivo* pre-treatment of rodents with cylophosphamide (CPA), a generalized inhibitor of immunoglobulin production by B cells, could also partially suppress this innate antiviral activity. CPA pre-treatment of rodents was found to decrease by more than half IgM blood concentration within 48 hours. In fact, when a single systemic dose of CPA was administered to rodents, significant

increases both in the number of animals with positively infected intracerebral tumors and in the propagation of viral infection throughout the brain neoplasm was observed after intra-arterial administration with hrR3. These studies thus suggested that transient inhibition of the innate antiviral response, that involved IgM and its likely interaction with complement, resulted in an augmented anticancer effect *in vivo*. This model thus would predict that *in vivo* pretreatment of rodents with agents that deplete complement, such as CVF, should also result in an augmented anticancer effect. Since one of CPA's action is to deplete plasma IgM while CVF depletes complement, the model also would predict that the combination of CPA and CVF would result in further augmentation of the anticancer effect of hrR3, delivered intra-arterially. Herein, we show that CVF does reverse the antiviral action of plasma *in vitro* against three different HSV strains and against a replication-defective adenovirus vector. *In vivo*, CVF pretreatment increases the number of positively infected tumor cells within a neoplastic intracerebral mass after intra-arterial administration of the oncolytic virus, hrR3. Addition of CPA appears to increase the propagation of virus within tumors and the combination of the two agents proves superior in its anticancer effects than other treatments. These results thus support a model of intravascular virus' infection and propagation within tumors that is initially modulated by the host innate antiviral response, which can be pharmacologically modified to further augment viral anticancer effects.

Results

Effects of rat and human plasma on viral vector infectivity. We sought to characterize the *in vitro* effect of undiluted plasma, harvested from immunocompetent and athymic rats as well as humans, on the infectivity of a retroviral, adenoviral, and HSV viral vectors expressing the *lacZ* reporter transgene. Both rodent and human plasma abolished the infectivity of adenovirus and HSV (Table 3). Human, but not rodent, plasma also inhibited the infectivity

of retrovirus. Further characterization of the effect of complement on the antiviral activity of plasma was obtained when mild heat treatment, shown to inactivate complement (Table 4), completely reversed the anti-adenoviral (Table 3) and partially reversed the anti-HSV activity of rodent plasma, respectively. There was no effect on the anti-adenoviral activity of human plasma, because this plasma is likely to possess neutralizing antibodies against the virus (Harvey, B.G., *et al., J. Virol. 73*:6729-6742 (1999); Schulick, A.H., *et al., J. Clin. Invest. 99*:209-219 (1997)), while there was partial reversal of the human anti-HSV and anti-retroviral activities. The pooled human plasmas selected for these studies was devoid of anti-HSV neutralizing antibodies, thus explaining the difference in antiviral activity upon heat inactivation. The effect of complement depletion on the antiviral activity of athymic rat plasma was much less than that of humans, which was less than that of immunocompetent rats. Taken in conjunction, these experiments suggested that both rodent and human complement inactivated HSV and adenoviral vectors. As previously shown, human complement also inactivated retroviral vectors (Welsh, R.M., *et al., J. Virol. 72*:4650-4656 (1998)). However, for HSV, complement-independent inactivation was also present, more so in athymic rats and naive humans than in immunocompetent rats.

Cobra Venom Factor (CVF), an in vivo depletor of complement, partially reverses the inactivation of HSV oncolytic viruses. We then sought to characterize the highest dilution of plasma, prepared from naive athymic rats, which would inactivate the HSV viral vector, hrR3. Figure 7 shows that plasma neutralized the virus at dilutions as high as 1:32 and that at lower dilutions this neutralization was almost complete. These results indicated that the antiviral action of plasma was present in relatively high concentrations.

To provide further experimental evidence for the *in vivo* contribution of complement to this activity, athymic rats were treated with cobra venom factor (CVF) whose C3 convertase action depletes complement levels in blood (Ballow, M. & Cochrane, C.G., *J. Immunol. 103*:944-952 (1969); Cochrane, C.G., *et al., J. Immunol. 105*:55-69 (1970); Hofmann, C. & Strauss, M., *Gene Ther. 5*:531-

536 (1998)). Plasma, harvested from these animals was not as effective in neutralizing hrR3 and reversal of antiviral activity was evident at a dilution of 1:8 (Figure 7). Taken in conjunction with the results described in Table 3, an *in vivo* role for complement inactivation of hrR3 appeared even more likely within the context of intravascular administration.

The inactivation of HSV by plasma and its reversal by CVF is not strain-dependent. Since HSV is known to escape complement's antiviral action through glycoprotein C binding of C3 (Lubinski, J.M., *et al., J. Virol. 72*:8257-8263 (1998)), it is possible that a previously unrecognized mutation in hrR3's gC gene was responsible for the apparent failure of immune evasion. We thus sought to determine the likelihood of this possibility, by testing the inactivation of two other different HSV viral vectors that express the lacZ reporter transgene: MGH1 and an HSV amplicon. While hrR3 was derived from KOS strain by another laboratory (Goldstein, D.J. & Weller, S.K., *J. Virol. 62*:2970-2977 (1988)), MGH1 was derived from F strain (Kramm, C.M., *et al., Hum. Gene Ther. 8*:2057-2068 (1997)) and the HSV amplicon was packaged from strain 17 by the authors (Cunningham, C. & Davison, A.J., *Virol. 197*:116-124 (1993); Saeki, Y., *et al., Hum. Gene Ther. 9*:2787-2794 (1998)). Figure 8 shows that inactivation of all three viral vector strains was observed with diluted (1:8), athymic rat plasma and that this inactivation was partially reversed if plasma from animals treated with CVF was employed for the assay. The finding that three different HSV vectors derived from three distinct HSV strains in two different laboratories displayed similar characteristics, suggested that secondary mutation(s) in gC were not likely responsible for the relative failure of HSV vector escape from complement's action.

Purified rat complement inhibits HSV vector infection of cells. As further proof of complement's ability to inactivate hrR3, the virus was incubated with purified rat complement in serum-free medium and then added onto cells in culture. Figure 9 shows that complement alone could partially inactivate the virus. Heat-inactivated plasma from athymic rats (diluted 1:4) also partially inactivated

the virus. Re-addition of complement to the heat-inactivated plasma produced greater than 95% inactivation. These findings thus indicated that rat complement provided a fairly effective barrier to hrR3 infection of cells.

CVF facilitates the transduction of multiple intracerebral tumors by the intravascular virus. To show that CVF pretreatment depleted complement activity in rodents, blood was collected from animals that two and three days earlier were injected with 20 and 60 units/kg of CVF, respectively. These dosages, this schedule, and the time of plasma harvesting were identical to the ones employed for the previous experiments and for subsequent experiments and did not result in clinical evidence of toxic side-effects. Complement's hemolytic activity in these animals was significantly reduced compared to controls (Table 4). These findings thus indicated that CVF effectively eliminated complement function in animals.

In previous studies, we and others had shown that the combination of intra-arterial hrR3 and disruptors of the BBB or BBTB, such as mannitol, bradykinin, or RMP7, would lead to infection of a single syngeneic or xenogeneic tumor established in rodent brains (Barnett, F.H., *et al., Cancer Gene Ther. 6*:14-20 (1999); Nilaver, G., *et al., Proc. Natl. Acad. Sci. USA 92*:9829-9833 (1995); Rainov, N.G., *et al., Hum. Gene Ther. 6*:1543-1552 (1995)). However, the number of positively transduced tumors was relatively low. Results described so far in this report indicated that animal pre-treatment with CVF might increase the survival of hrR3 in rodent plasma and thus facilitate the transduction of an intracerebral tumor. In order to show the therapeutic power of this approach, we employed an animal model in which three separate and distinct tumor masses were established in the animal's brain. Athymic rats harboring three large and separate glioma tumors were thus pre-treated with CVF (or saline) the day before and the day of intra-arterial injection with hrR3 (or mock) plus RMP7 (or vehicle). The anatomic extent of lacZ gene delivery within the 3 separate neoplasms was then measured two days later. Table 5 shows that complement depletion by CVF treatment of animals significantly facilitated the transduction of three separate

intracerebral turnors by intra-arterial hrR3. These findings thus confirmed that CVF pre-treatment of rodents led to an increase in the number of positively transduced intracerebral tumors, likely through its depletion of complement.

CPA enhances the action of CVF. The previous results showed that there was a significant increase in the number of transduced tumors two days after treatment with CVF and hrR3 (Table 5). However, when tumors were assayed for lacZ cDNA expression four days later, no further increases in the number of tumors nor increases in the area of intraneoplastic plaques (a marker of viral propagation) were observed (Table 6 and Figure 10). We had previously shown that neutralizing (elicited) humoral responses could inhibit the propagation of hrR3 within transduced brain neoplasms; cyclophosphamide (CPA) pretreatment of rats partially suppressed these responses, permitting efficient and reliable oncolytic effects against multiple tumors by an intra-arterially administered virus (Ikeda, K., *et al., Nature Med. 5*:881-887 (1999)). We thus sought to demonstrate if CVF and CPA would act in concert to anatomically increase the volume of tumor infected by the virus. For this experiment, CPA had to be administered two days after injection of hrR3 and CVF because same day administration produced significant animal mortality. This regimen did result in a significant increase in the percent infection of the three intracerebral tumors by hrR3 in animals exposed to CVF followed two days later by CPA (Table 6). These results suggested a mechanism where the primary effect of CVF was to initially deplete complement, enhancing the initial infection of tumor cells by intravascular virus, while one of the actions of CPA was to inhibit both innate and elicited neutralizing humoral antiviral responses. This may have allowed for further viral propagation into the neoplasm.

Histologic analyses of brains harvested from treated animals provided some evidence for this mechanism. Figure 10 shows that CVF-treated animals harbored small lacZ-positive "plaques" within tumors, 2 and 4 days after intra-arterial administration of hrR3, while control animals did not. Addition of CPA to the CVF treatment caused a significant increase in the size of lacZ-positive

"plaques" that now appear to occupy the majority of each tumor. In fact, by day 8, involution and disappearance of tumors has occurred. These results thus confirm the facilitating action of CVF and CPA in providing for initial transduction and subsequent propagation of the oncolytic virus within a neoplastic mass.

Survival analysis. We then sought to characterize the anticancer effect of intra-arterial hrR3 after depletion of complement by CVF and inhibition of elicited and innate humoral responses by CPA. Figure 11 shows that the combination of intra-arterial hrR3, CVF and CPA significantly increased the survival of athymic rats, harboring three separate intracerebral human glioma xeno-grafts, compared to other treatments. Addition of RMP7 to this regimen increased this effect further, but not in a statistically significant fashion. These results thus suggested that strategies aimed at partially suppressing innate (complement and preimmune Ig) and elicited (neutralizing Ig) antiviral responses can significantly increase the oncolytic efficacy of an intravascular viral vector against multiple brain tumors.

Toxicity results. Although intra-arterial delivery can provide a route to target multiple tumor masses within an organ and inactivation of complement and humoral responses provided evidence of efficacy, it was important to determine if systemic infection with a replicating virus occurred in animals. There was no evidence of reporter transgene expression or viral antigen expression in the liver, lung, spleen, kidney or brain of treated animals, four days after intra-arterial administration of hrR3. PCR analysis revealed hrR3 viral genomes only in the brain tumor, but not in contralateral brain, lung, liver, spleen, or kidney. However, when analyzed by Southern blotting, presence of hrR3 genomes was evident in all analyzed organs (Figure 12). This result thus showed that hrR3 genomes had established within cells of these organs, although there was no evidence of active viral gene expression and replication within them..

Materials and Methods

Vector stocks. hrR3 is the genetically engineered HSV mutant, derived from HSV-1 KOS, which has an intact TK gene and a disruption of the UL39 gene through insertion of the Escherichia coli lacZ gene under the control of the ICP6 promoter (Goldstein, D.J. & Weller, S.K., *J. Virol. 62*:2970-2977 (1988)). Viral stocks were generated in African green monkey kidney cell culture (Vero) and titered by plaque assays. As a control, a mock-infection extract was prepared from mock-infected cells using the same procedures. MGHl is a second-generation replication-conditional herpes simplex virus type I (HSV) vector defective for both ribonucleotide reductase (RR) and the neurovirulence factor γ34.5 (Kramm, C.M., *et al., Hum. Gene Ther. 8*:2057-2068 (1997)). The helper-free HSV amplicon (pHSVlac) has been described (Geller, A.I. & Freese, A., *Proc. Natl. Acad. Sci. USA 87*:1149-1153 (1990)) and it consists of a plasmid bearing: a) the HSV1 origin of DNA replication, b) the "Pac" sequence to support packaging, and c) an IE4/5 promoter driving lacZ. This amplicon is packaged in Vero cells by cotransfection with a set of five cosmids representing the entire HSV1 genome, but lacking the "Pac" sequences needed for DNA cleavage/packaging (Cunningham, C. & Davison, A.J., *Virol. 197*:116-124 (1993)). This allows for packaging of the amplicon plasmids without recombination and packaging of wild-type HSV or helper virus. The adenovirus vector was obtained originally from Drs. Alan Smith and Bruce Roberts (Genzyme) and it possesses: a) a deletion in the ElA/ElB region, b) an intact E3 region, and c) a modified E4 region, in which the entire E4 locus has been deleted and E4 ORF and protein IX have been reinserted. A CMV promoter-lacZ gene cassette has also been inserted into the El locus.

Cell culture. African green monkey kidney (Vero) cells were purchased through the America Tissue Culture Collection. Human U87dEGFR glioma cells were a generous gift of Dr. H.-J. Su Huang (University of California at San Diego). This cell line was established by retroviral transfer of a mutant epidermal

growth factor receptor (de 2-7 EGFR) into U87 human glioblastoma cell line, enhancing its tumorigenic capacity in the brain of nude mice (Nagane, M., *et al.*, *Cancer Res. 56*:5079-5086 (1996)). U87dEGFR cells were propagated at 37°C in an atmosphere containing 5% carbon dioxide in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal calf serum containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml G418 (Sigma).

Assays for plasma antiviral activity. Rat or human plasma were serially diluted with PBS. One hundred μl of plasma was incubated with 2 x 10^4 pfu of viral vectors for 1.5 hours at 37°C and then applied onto 4 x 10^4 Vero cells in 24-well plates. Sixteen hours later, cells were fixed and stained for β-galactosidase activity. In pilot experiments, lacZ transduction of cells was essentially identical to the number of *lacZ*-expressing plaques measured 5-7 days later. For some experiments, blood samples were obtained from rats that had been injected intraperitoneally with cobra venom factor (CVF; Quidel, San Diego, California) at a dose of 60 U/kg and 20 U/kg, 3 and 2 days before plasma preparation. For studies employing rat complement (Accurate Chemical and Scientific Co., Westbury, NY) virus was pre-incubated with HBSS, complement (3 mg/ml) in HBSS, heat-inactivated plasma prepared from athymic rats (diluted 1:4), or complement re-added to heat-inactivated plasma to a concentration of 3 mg/ml. The physiologic concentration of complement is 3.6 mg/ml.

Animal studies. Adult female nude rats (rnu/rnu) were anesthetized with an intraperitoneal injection of 0.5 ml of 0.9% NaCl containing 12.5 mg of ketamine and 2.5 mg of xylasine. After immobilizing the rats in a stereotactic apparatus and placing a linear skin incision over the bregma, burr holes (1 mm in diameter) were drilled in the skull approximately 1 mm anterior to and 2 mm lateral to the bregma on both sides, and 3 mm posterior and 2 mm lateral to the bregma on the right side. Two hundred thousand U87dEGFR cells (in a 2 μl volume) were injected at a depth of 3.5 mm from the dura, using a 5 μl Hamilton syringe.

Six days later, CVF (60U/kg) was injected intraperitoneally. The next day, CVF (20U/kg) administration was repeated, followed by intra-arterial catheterization with hrR3 and/or RMP7 (Alkermes, Inc., Cambridge, MA). In some animals, a single intraperitoneal dose of cyclophosphamide (100 mg/kg) was also administered, two days after the last dose of CVF and the intra-arterial delivery of hrR3 and RMP7. The catheterization technique used was essentially identical to the one previously described (Rainov, N.G., *et al., Hum. Gene Ther. 6*:1543-1552 (1995)). Briefly, RMP-7 at a dose of 1.5 pg/kg or vehicle was infused over 10 min. Midway through the infusion, a 200 µl bolus of virus (2 x 10^9 pfu) or vehicle was given. For tumor transduction assays, animals were sacrificed at different days after catherization and then perfused by intracardiac infusion of a solution containing 4 % neutral paraformaldehyde (PFA) in 0.9% sodium chloride and 10 mM sodium phosphate, pH=7 (PBS). After harvesting, brains were transferred to 30% sucrose in PBS for 2 days, frozen over liquid nitrogen and stored at – 80 °C. For survival studies, rats were observed twice daily until they exhibited neurologic impairment (inability to feed, drink, or move) at which time they were euthanatized. Survival analysis was performed employing the statistical software Microsoft Excel using Kaplan-Meier survival estimation and the Wilcoxon test for significance.

Histochemistry for virus distribution assay. Brains and their tumors were analyzed by sectioning (20 μm thick) on a cryostat and air-dried at room temperature. Sections were stained by histochemistry using the X-Gal substrate, as previously described (Boviatsis, E.J., *et al., Hum. Gene Ther. 5*:183-191 (1994)) and were counter-stained with neutral red.

Quantitative analysis of lacZ cDNA expression after virus injection. Three randomly selected sections of brain tumors were selected from each animal and were analyzed using an Olympus BX60 microscope. The sections were scanned by Sony 3-chip Color Video Camera at 20x magnification and the entire tumor area and β-galactosidase positive area were measured using Image Pro Plus

Imaging Software. Selection of sections and scanning was performed by an observer (S.J.), blinded to the identity of the sections.

Analysis of viral genomes. Two days after oncolytic virus administration, animals were sacrificed and genomic DNA prepared from the brain tumor, brain separate from the tumor, lung, liver, spleen, and kidney. Polymerase chain reaction (PCR) analysis was performed using primers specific for hrR3. The 5'-primer hybridizes to the 5'-region of the HSV ICP6 genome and it is composed of sequence 5'-GAG GAC GAC TTT GGG CTT CT-3' (SEQ ID NO:1). The Y-primer hybridizes to the 5'-region of the *lacZ* cDNA, inserted in the ICP6 region of hrR3(Goldstein, D.J., *J. Virol. 62*:2970-2977 (1988)), and its sequence is 5'-TCC CAC GCC ATC CCG CAT CT-3' (SEQ ID NO:2). The resulting amplified product measures approximately 1000 base pairs in length. After agarose gel electrophoresis, ethidium bromide staining, and transfer to nitrocellulose filters, Southern analysis of the PCR products was performed using a *lacZ* cDNA probe, that hybridizes to the amplified PCR fragment. The enhanced chemiluminescence system (ECL, Amersham) was employed for non-radioactive probe labeling.

Discussion

The primary objective of this report was to study the interaction of complement with viral vectors exposed to plasma. Delivery of such vectors through the vasculature may potentially target multiple and distinct tumor foci within an organ such as the brain. Because current gene- and viral-based therapies are commonly administered as intratumoral injections, they will necessarily remain limited to a local antitumor effect, unless they elicit systemic anticancer immunity. Findings presented in this report demonstrate that: 1) Factors present in rat and human plasma are powerful inhibitors of viral vector infection of cells, 2) One of the components in plasma responsible for this activity is complement, 3) *In vivo* depletion of complement facilitates infection by an intra-arterial HSV of tumor

cells located within three separate intracerebral neoplasms, 4) This depletion is not sufficient to allow for increased propagation of virus within tumors and additional treatment with cyclophosphamide is needed to achieve anatomically extensive infection and propagation of oncolytic virus within tumors. These findings are relevant to understanding the interaction between oncolytic virus and the innate and elicited humoral immune response and how this interaction governs the process of viral infection of tumors and the subsequent propagation of progeny virions within the neoplastic mass.

The lytic ability of HSV is being harnessed as a novel cancer therapy. The primary issue that will affect the use of this virus as a clinically relevant anticancer agent are safety and efficacy: 1) Safety directly depends on the replication selectivity of the mutant virus. Several strategies exist to render HSV replication selective for tumor cells. Viral genes, needed for viral replication in postmitotic cells, can be deleted or mutated. Such genes encode enzymes that regulate nucleic acid metabolism in infected cells, to allow for viral DNA synthesis (Boviatsis, E.J., *et al., Gene Ther. 1*:323-331 (1994); Martuza, R.L., *et al., Science 252*:854-856 (1991); Pyles, R.B., *et al., Hum. Gene Ther. 8*:533-544 (1997)). Another approach consists of deleting viral genes responsible for regulating viral progeny production usually by modulation of the infected cell's apoptotic response (Chambers, R., *et al., Proc. Natl. Acad. Sci. USA 92*:1411-1415 (1995); Kesari, S., *et al., Lab. Invest. 73*:636-648 (1995); Markert, J.M., *et al., Neurosurg. 32*:597-603 (1993)). A third approach consists of using tumor-specific promoters to regulate expression of essential viral genes (Chung, R., *et al., J. Virol. 73*:7556-7564 (1999)). A fourth approach consists of using altering the receptor specificity of HSV glycoproteins toward tumor rather than normal tissue (Laquerre, S., *et al., J. Virol. 72*:9683-9697 (1998)). It is likely that a combination of these approaches may ultimately generate a series of oncolytic HSV that are extremely selective in their targeting of tumor cells; 2) Efficacy depends on the ability of the virus to efficiently infect tumor cells and propagate within infected neoplastic masses. Results presented in this paper and in an additional publication (Ikeda, K., *et al.,*

Nature Med. 5:881-887 (1999)) suggest that the mechanism of CPA action is not only as a direct antitumor agent but also as a facilitator of virus survival and propagation within infected tumors. The efficacy of an oncolytic HSV can also be augmented through its ability to deliver additional anticancer functions in infected cells. For instance, gene that encodes prodrug-activating enzymes can be engineered into the viral genome to combine a viral oncolytic and a chemotherapy-sensitizing effect (Boviatsis, E.J., *et al., Cancer Res.* 54:5745-5751 (1994); Boviatsis, E.J., *et al., Gene Ther.* 1:323-331 (1994)). We have recently shown that an oncolytic HSV can be used to deliver the CPA-susceptibility transgene, CYP2B1, thus producing an enhanced anticancer effect (Chase, M., *et al., Nat. Biotechnol.* 16:444-448 (1998)). These anticancer effects can be further enhanced by the addition of prodrug-activating genes that will pharmacologically synergize with CPA/CYP2B1 gene therapy (Aghi, M., *et al., Cancer Res.* 59:3861-3865 (1999)).

Our findings show that pre-immune plasma from human and rats inactivates both replication-conditional and defective HSV. Furthermore, human plasma (not pre-screened for adenoviral antibodies) and rat plasma also inactivated a replication-defective adenoviral vector. In rats, pharmacologic or physical depletion of complement almost completely reversed this inactivation for adenovirus and partially reversed it for HSV. Our published results indicate that complement is not the only innate anti-HSV factor in rat plasma. In fact, antibody-mediated depletion or neutralization of pre-immune plasma IgM also leads to a partial reversal of antiviral activity and it synergizes with complement depletion to block innate anti-HSV action (Ikeda, K., *et al., Nature Med.* 5:881-887 (1999)). Quantitatively, assuming that a rat's blood volume is approximately 20 ml and that we have saturated antiviral activity, then our assay would predict that at the highest dilution of plasma that still possesses antiviral activity (1:32), approximately 2×10^6 pfus of hrR3 would be eliminated and thus with undiluted plasma approximately 6.4×10^7 pfus of hrR3 would be eliminated as a single bolus. The observed inability of an intra-arterial bolus of 10^9 pfus of hrR3 to

efficiently infect a brain neoplasm is quantitatively rational, taking into account that: A) Over a 24 hour time period circulating and/or infecting virus will be exposed to much higher volumes of plasma, B) Physical barriers (such as the blood-brain, blood-brain-tumor barrier, splenic and hepatic trapp ng of circulating substances) will limit viral penetration into the tissue and organ, and C) Innate antiviral responses, mediated by NK cells, neutrophils, and macrophages will also inactivate the virus.

It thus appears that a threshold or "innate" barrier exists to oncolytic virus' infection of turnors and one would predict that circumvention of this barrier requires either depicting one or more of its components or increasing the dose of injected virus. Published and currently presented experimental evidence does agree with this model. Evidence in this and another report shows that the innate barrier can be lowered by depleting complement and/or IgM using physical (heat) or phannacologic (CPA and/or CVF) methods. Conversely, when higher doses of hrR3 (10 instead of 10^9) were delivered intravascularly, an increase in anticancer efficacy was also observed (Rainov, N.G., *et al., Cancer Gene Ther. 5*:158-162 (1998)). These results thus demonstrate that methods exist to overcome the initial innate barrier and allow for infection of multiple tumors in the brain.

With adenovirus, we employed human plasma that is likely to contain adenoviral neutralizing antibodies, present in greater than one third of adult humans (Schulick, A.H., *et al., J. Clin. Invest. 99*:209-219 (1997)). Even when complement was depleted, human plasma effectively inhibited the adenoviral vector, by complement-independent humoral responses. Recently, results from a human phase I trial where an adenoviral vector was instilled in the pleural cavity of patients afflicted with malignant mesothelioma were described in relation to the impact of pre-existing titers of neutralizing antibodies (Molnar-Kimber, K.L., *et al., Hum. Gene Ther. 9*:2121-2133 (1998)). Although gene transfer was still observed, a comparison with a group of patients that did not have pre-existing titers of neutralizing antibodies does reveal a significant difference in terms of gene

transfer in favor of the latter group. Furthermore, it is likely that instillation of the vector in the pleural cavity provides a less favorable milieu to humoral neutralization than direct exposure of the vector to blood and its components, as in our described experiments.

CVF specifically depletes the C3 component of complement (Cochrane, C.G., *et al., J. Immunol. 105*:55-69 (1970)). The low doses employed in this report did not result in significant toxicity in rodents, but clearly facilitated the initial infection by intra-arterial oncolytic HSV of three tumor masses in rodent brains. CVF is not known to have other effects on the blood-brain-barrier and is commonly and successfully used in animal models of xenotransplantation, in order to avert the hyperacute rejection reaction, caused by natural antibodies and complement (Leventhal, J.R., *et al., Transplantation 55*:857-865, discussion 865-866 (1993); Suh, C.H., *et al., J. Invest. Surg. 10*:37-40 (1997)). In this regard, pharmacologic and monoclonal humoral anti-cornplement compounds are being developed and tested in clinical trials in humans to circumvent hyperacute immune rejection of xeno- and allo-transplants (Candinas, D., *et al., Transplantation 62*:1-5 (1996); Candinas, D., *et al., Transplantation 62*:336-342 (1996); Ferran, C., *et al., Blood 91*:2249-2258 (1998); Koyamada, N., *et al., Transplantation 62*:1739-1743 (1996); Kroshus, T.J., *et al., Transplantation 60*:1194-1202 (1995); Miyatake, T., *et al., Transplantation 65*:1563-1569 (1998); Soares, M.P., *et al., Nat. Med. 4*:1073-1077 (1998); Soares, M.P., *et al., J. Immunol. 161*:4572-4582 (1998)). The results in this report tend to argue that intravascular, oncolytic HSV treatment of tumors will also be limited by a similar hyperacute rejection of the viral vector and of the initially infected tumor cells and that further clinical development of this approach may require the use of pharmacologic or monoclonal humoral methods to avert the innate immune response.

We showed that CVF increased the number of initially infected tumors and tumor cells, two days after intravascular administration of oncolytic virus. However, four days later, there was no increase (and, in fact, by comparing the results in Table 5 versus those of Table 6, there might even have been a decrease)

in the area of tumor transduction and in the size of the viral "plaques" within the tumors (compare the CVF-treated brains of Figure 10A with those of Figure 10B). This indicated that initial depletion of complement was not sufficient to allow for viral propagation. Previously, we had shown that CPA, while not depleting complement levels, inhibited complement antiviral function through the classical activation cascade, perhaps through depletion of rodent IgM levels, and at later time points (four days), inhibited the rise in neutralizing antibody titers. This resulted in extensive transduction of tumors and facilitated viral propagation within infected tumors. This suggested that CVF and CPA in concert could effectively suppress complements' ability to limit viral infection of tumors and that CPA would then eliminate the neutralizing humoral response that limited viral propagation within tumors. Results in this report do seem to support this model: The combination of the two agents was superior than either alone and CPA significantly augmented the CVF-effect at the four day time point. We thus propose a model, where initial infection of brain tumors by intravascular virus limited by complement (and other blood components), can be circumvented by either depleting it through CVF or by inhibiting its function (through CPA). However, further propagation of virus from initially infected tumor cells becomes limited by the formation of neutralizing antibodies, a process that is abrogated by CPA (Ikeda, K., *et al., Nature Med.* 5:881-887 (1999)).

While the approach described in this report showed evidence of efficacy, as evidenced by the significant prolongation in the survival of animals, harboring three intracerebral neoplasms, questions related to its safety do arise. In athymic rodents, we did not find evidence of oncolytic HSV replication in tissues other than the glioma, as evidenced by lack of reporter transgene or HSV antigen expression. However, we did find viral genomes in cells from these organs, showing that infection of these tissues did occur. Since we are employing rodents for these studies, whose cells are notoriously impervious to HSV infection and replication, the definition of toxicity in rodents is not very reliable. Intravascular administration of a suitable oncolytic HSV in primate models of HSV toxicity will

provide a more reliable measure of safety (Hunter, W.D., *et al.*, *J. Virol. 73*:6319-6326 (1999)).

The ability to infect, transduce, and lyse multiple tumors within an organ such as brain provides a first step towards rendering gene- or viral-based therapies useful applications for illnesses that are not currently treatable. Further elucidation of the mechanisms of viral passage from the vascular spaces into the tumor and of effects of the early immune responses against the virus and virally-infected cells can provide refinement in this therapeutic strategy. If limited and transient manipulation of these innate and early humoral responses without alterations of more prolonged cellular responses provides an effective anticancer effect, it is possible that toxicity from a prolonged viral infection in tumors may be avoided.

Table 3. The *in vitro* effect of control and heat-treated (complement-depleted) plasma from rats and humans on viral vector infectivity of cells.

Immunocompetent rat[A]			Athymic rat		Human	
Heat-treat.[B]	-	+	-	+	-	+
Adeno.[C]	0.8(0.2)[D]	98.1(3.1)	0	95.2(8.4)	0	0
Retro.	97(4.5)	104(4)	98.5(6.6)	102(4)	20(1.6)	68.4(11.2)
HSV	0	21.2(3.6)	0	0.06(0.02)	0.02(0.01)	7.8(0)

[A]Plasma was prepared from immunocompetent and athymic rats as well as humans, as described in the Methods section.

[B]Plasma was exposed to a temperature of 56°C for 30 minutes.

[C]An adenovirus, retrovirus, or HSV vector expressing the *lacZ* cDNA was pre-incubated for 1.5 hours with plasma before adding onto cells. Sixteen hours later, the number of lacZ cDNA-expressing cells was recorded.

[D]Values represent the mean percentage of lacZ cDNA-expressing cells from triplicate dishes with parenthetical values representing the standard error of the mean.

Table 4. Complement function in athymic and immunocompetent rats.

	Athymic	Immunocompetent
Control[A]	393[B]	396.5
Heat treatment[C]	1	1
Cobra Venom Factor[D]	35	ND[E]

[A]Control plasma was prepared from rodents as described in Methods.

[B]Values represent the average from two animals, as assayed by units per milliliter of 50% complement's hemolytic activity.

[C]Plasma from control rodents was exposed to 56°C for thirty minutes.

[D]Athymic rats were injected with Cobra Venom Factor at 60 and 20 U/Kg on two successive days. Forty-eight hours after the last injection, plasma was prepared as described in methods.

[E]Not determined.

Table 5. A summary of *lacZ* cDNA transduction in each of three distinct intracerebral tumors, two days after intravascular administration of hrR3 in the presence or absence of CVF and RMP7.

Rat No.	CVF	RMP7	% intraneoplastic *lacZ* transduction[A]		
			Tumor 1[B]	Tumor 2	Tumor 3
1	+	+	2.3 [C]	3.5	1.4
2	+	+	3.8	2.5	0.2
3	+	+	0	3.9	3.8
4	+	+	0	3.4	7.2
5	+	-	4.4	2	7.1
6	+	-	0	0	0
7	+	-	0	2.4	1.7
8	-	+	2.3	0	0
9	-	+	0	0	0
10	-	+	0	0	0
11	-	-	0	0	0
12	-	-	0	0	0
13	-	-	0	0	0

[A]Percentages were calculated by computer-assisted analysis of lacZ-expressing tumor area, two days after intravascular administration of hrR3, divided by the total tumor area.

[B]Tumors 1, 2, and 3 denote the right frontal, right thalamic, and left frontal neoplasms, respectively.

[C]Values represent the mean transduced tumor area from five randomly selected sections analyzed in a blinded fashion by S.J.

Table 6. A summary of *lacZ* cDNA transduction in each of three distinct intracerebral tumors, four days after intravascular administration of hrR3 in the presence or absence of CVF and CPA [A].

Rat No.	CVF	RMP7	% intraneoplastic *lacZ* transduction [B]		
			Tumor 1 [C]	Tumor 2	Tumor 3
1	+	+	92.1 [D]	75.5	76.2
2	+	+	29	42.2	53.1
3	+	+	68.9	75.4	0
4	+	-	0	0.7	1.4
5	+	-	0.2	0	0
6	+	-	1.9	0	1.7
7	-	+	45.1	0	0
8	-	+	0	0	0
9	-	+	0	0	0
10	-	-	0	0	0
11	-	-	0	0	0
12	-	-	0	0	0

[A]All animals were treated with RMP7. CPA was administered two days after the intravascular injection of hrR3 and the second injection of CVF.

[B]Percentages were calculated by computer-assisted analysis of lacZ-expressing tumor area, two days after intravascular administration of hrR3, divided by the total tumor area.

[C]Tumors 1, 2, and 3 denote the right frontal, right thalamic, and left frontal neoplasms, respectively.

[D]Values represent the mean transduced tumor area from five randomly selected sections analyzed in a blinded fashion by S.J.

All documents, e.g., scientific publications, patents and patent publications recited herein are hereby incorporated by reference in their entirety to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference in its entirety.

What Is Claimed Is:

1. A method of administering a genetic vector to a target cell in a patient, said method comprising:

(a) immunosuppressing said patient; and

(b) administering said genetic vector to said patient.

2. The method of claim 1, further comprising:

(c) administering to said patient a blood-organ barrier modifier in a pharmaceutically effective amount to disrupt a blood-organ barrier in said patient.

3. The method of claim 1, further comprising:

(d) administering a complement inhibitor to said patient.

4. The method of claim 2, further comprising:

(d) administering a complement inhibitor to said patient.

5. The method of claim 1, wherein said method is for treatment of a neoplasm.

6. The method of claim 5, wherein said neoplasm is selected from the group consisting of astrocytoma, oligodendroglioma, neurofibroma, glioblastoma, ependymoma, Schwannoma, neurofibrosarcoma, meningioma, medulloblastoma, and metastatic cancer.

7. The method of claim 1, further comprising:

(e) administering to said patient a blood-brain barrier modifier in a pharmaceutically effective amount to disrupt a blood-brain barrier in said patient.

8. The method of claim 7, further comprising:

(d) administering a complement inhibitor to said patient.

9. The method of claim 7, wherein said method is for treatment of a disease selected from the group consisting of inborn error of metabolism, neurodegenerative disorder, stroke, tumor, spinal cord trauma, and nerve trauma.

10. The method of claim 7, wherein said target cell is a brain cell.

11. The method of claim 1, wherein said target cell is selected from the group consisting of a brain cell, eye cell, gastrointestinal cell, heart cell, intestinal cell, kidney cell, liver cell, lung cell, kidney cell, respiratory cell, testicular cell, prostatic cell, breast cell, and bone cell.

12. The method of claim 1, wherein said patient is immunosuppressed by administering a pharmaceutically effective amount of an agent selected from the group consisting of azathioprine, cobra venom factor, cyclosporin A, FK506, FK520, ganciclovir, cyclophosphamide, ifosfamide and 5-fluorouracil.

13. The method of claim 2, wherein said blood-organ barrier modifier is selected from the group consisting of a bradykinin agonist, osmotic agent, cyclic GMP modulator, cytokine, nitric oxide modulator, by physical method, and combinations thereof.

14. The method of claim 2, wherein said blood-organ barrier modifier is RMP-7.

15. The method of claim 1, wherein said genetic vector is a viral vector derived from a virus selected from the group consisting of herpes virus,

adenovirus, retrovirus, lentivirus, cytomegalovirus, varicella zoster virus, psuedorabies, adeno-associated virus, Epstein-Barr virus, and hybrid virus.

16. The method of claim 3, wherein said complement inhibitor is selected from the group consisting of soluble complement receptor 1, soluble complement receptor 1 deleted in C4 binding region, antibody against C5, specific isocoumarin inhibitor of factor B and factor D, inhibitor of C2, C1 inhibitor, and SDZGPI 562, SUT 175, and K76.

17. A method of administering a genetic vector to a target cell in a patient, said method comprising:

(a) administering a complement inhibitor to said patient; and

(b) administering said genetic vector to said patient.

18. The method of claim 17, further comprising:

(c) administering to said patient a blood-organ barrier modifier in a pharmaceutically effective amount to disrupt a blood-organ barrier in said patient.

19. The method of claim 17, wherein said method is for treatment of a neoplasm.

20. The method of claim 19, wherein said neoplasm is selected from the group consisting of astrocytoma, oligodendroglioma, neurofibroma, glioblastoma, ependymoma, Schwannoma, neurofibrosarcoma, meningioma, medulloblastoma, and metastatic cancer.

21. The method of claim 18, wherein said blood-organ barrier modifier is selected from the group consisting of a bradykinin agonist, osmotic agent, cyclic

GMP modulator, cytokine, nitric oxide modulator, by physical method, and combinations thereof.

22. The method of claim 21, wherein said blood-organ barrier modifier is RMP-7.

23. The method of claim 17, wherein said genetic vector is a viral vector derived from a virus selected from the group consisting of herpes virus, adenovirus, retrovirus, lentivirus, cytomegalovirus, varicella zoster virus, psuedorabies, adeno-associated virus, Epstein-Barr virus, and hybrid virus.

24. The method of claim 17, wherein said complement inhibitor is selected from the group consisting of soluble complement receptor 1, soluble complement receptor 1 deleted in C4 binding region, antibody against C5, specific isocoumarin inhibitor of factor B and factor D, inhibitor of C2, C1 inhibitor, and SDZGPI 562, SUT 175, and K76.

25. A method of administering a genetic vector to a brain cell in a patient, said method comprising:

(a) immunosuppressing said patient; and

(b) administering said genetic vector to said brain cell of said patient.

26. The method of claim 25, further comprising:

(c) administering to said patient an agent, which increases blood volume in the brain of said patient, in a pharmaceutically effective amount to disrupt a blood-brain barrier in said patient.

27. The method of claim 25, further comprising:

(d) administering a complement inhibitor to said patient.

28. The method of claim 25, wherein said method is for treatment of a brain tumor.

29. The method of claim 28, wherein said brain tumor is selected from the group consisting of astrocytoma, oligodendroglioma, and glioblastoma.

30. The method of claim 26, wherein said agent, which increases blood volume in brain of said patient, is a blood-brain barrier modifier selected from the group consisting of a bradykinin agonist, osmotic agent, cyclic GMP modulator, cytokine, nitric oxide modulator, by physical method and combinations thereof.

31. The method of claim 30, wherein said agent, which increases blood volume in the brain of said patient, is a bradykinin agonist selected from the group consisting of bradykinin and RMP-7.

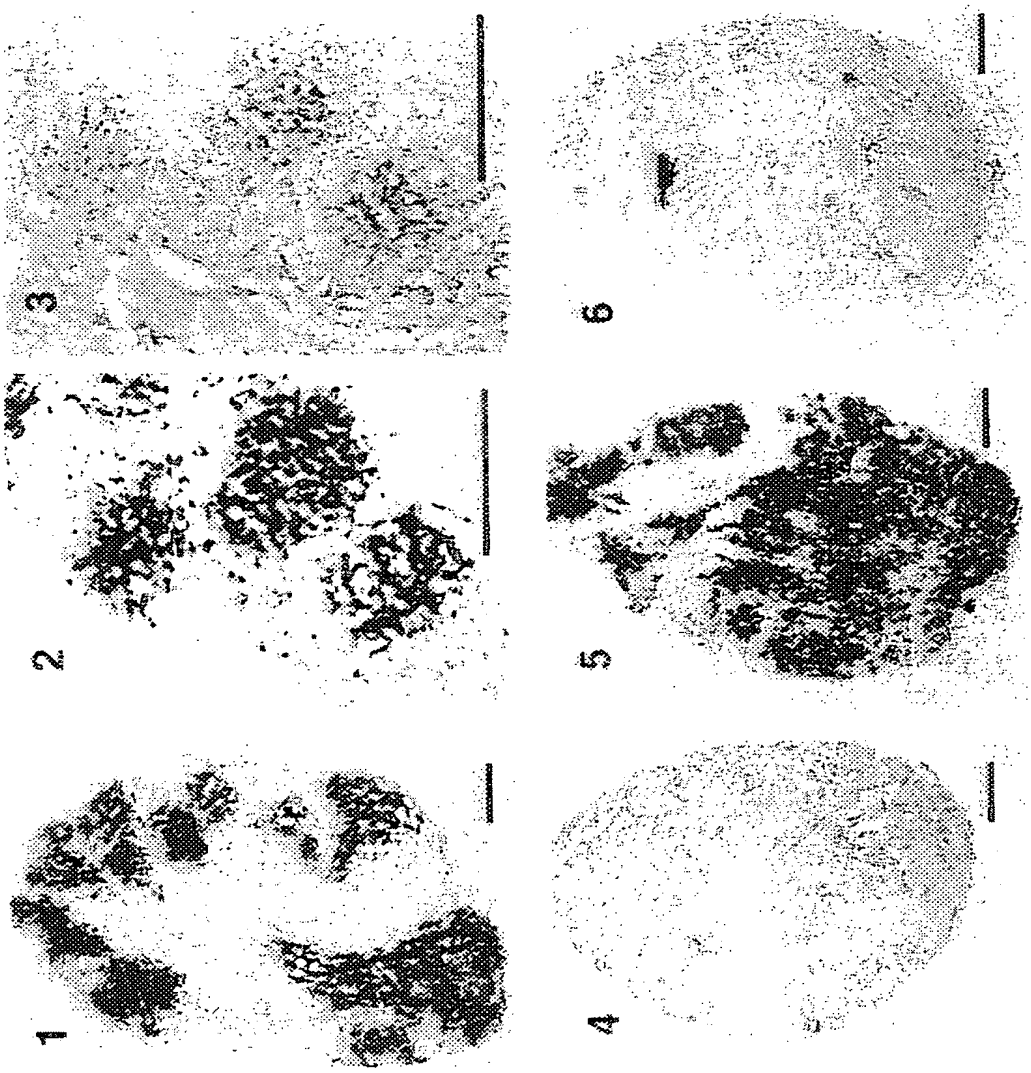


FIG. 1A

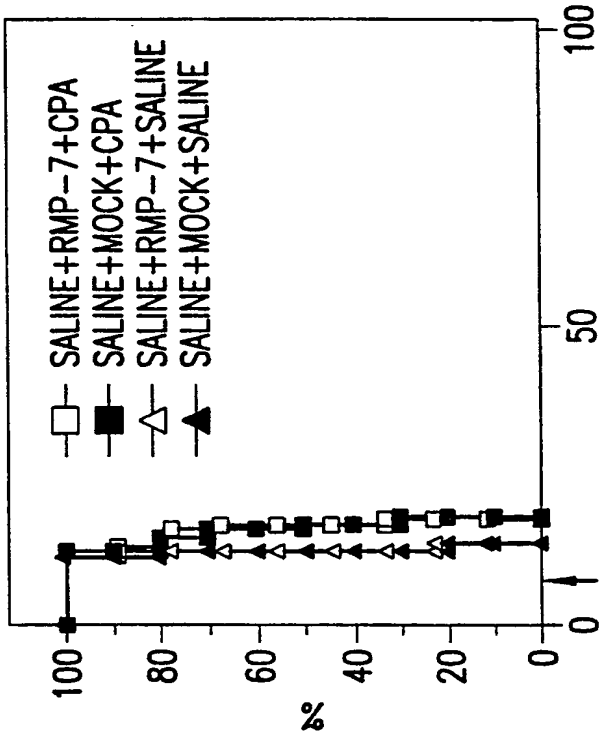


FIG. 1B-1

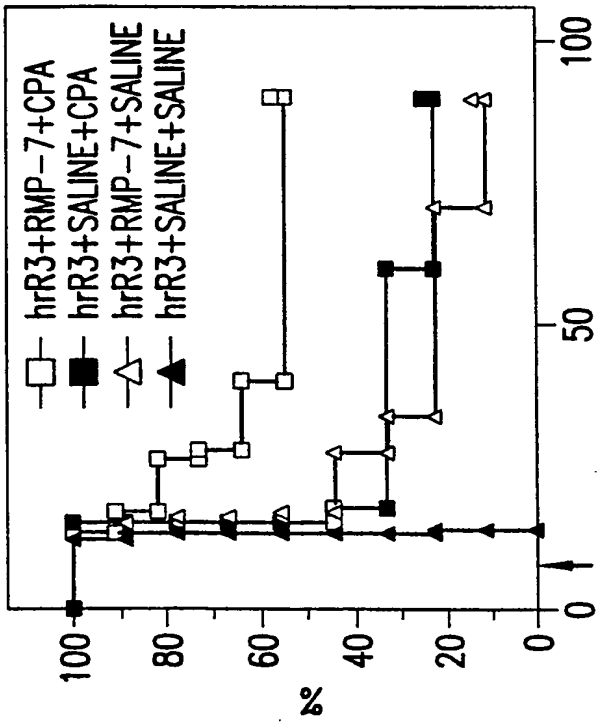


FIG. 1B

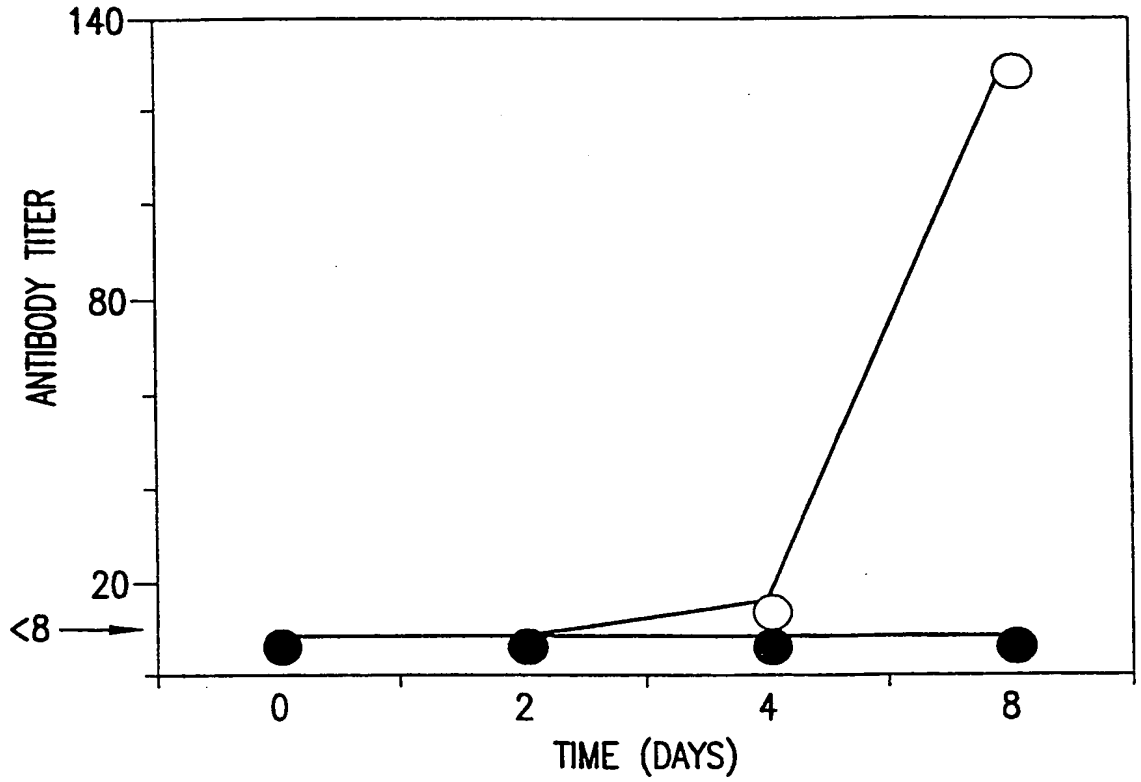


FIG. 1C

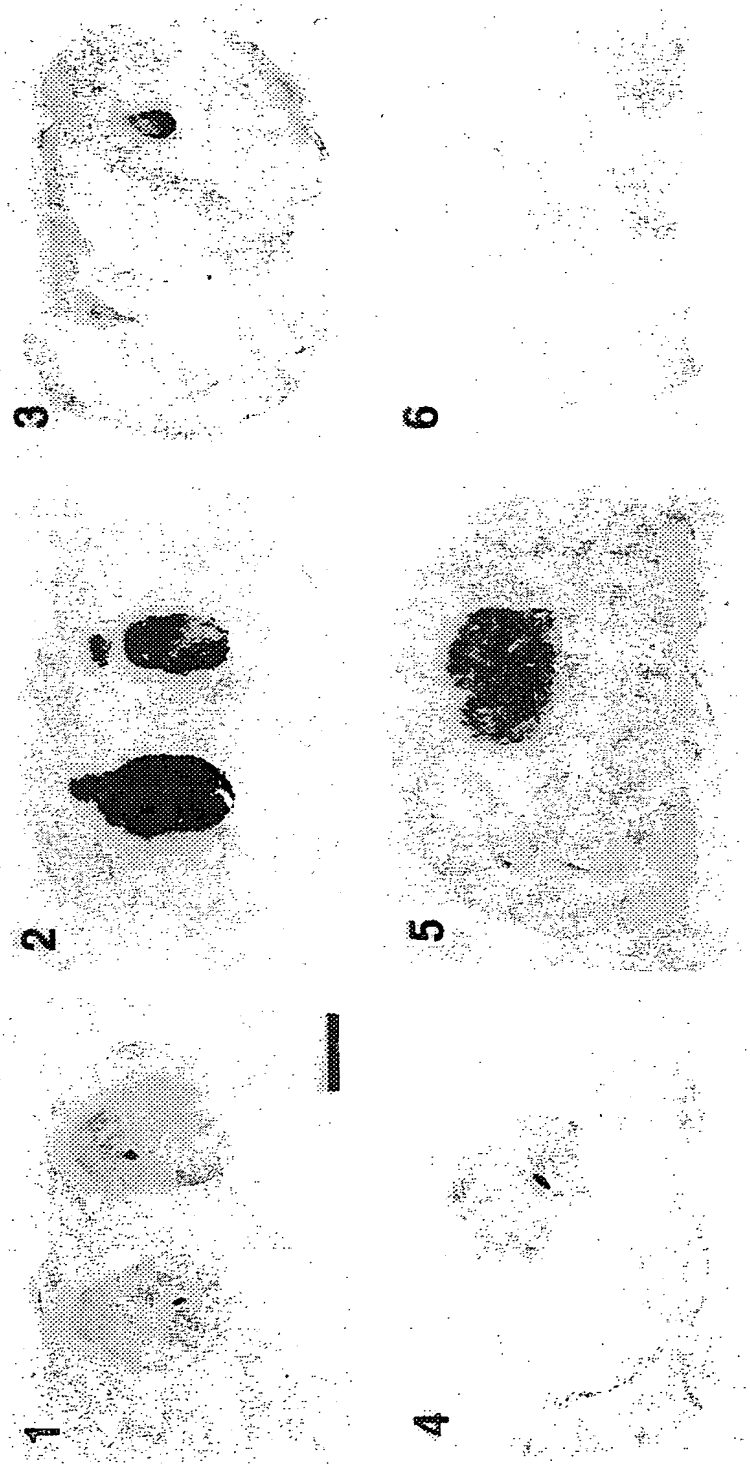


FIG.2A

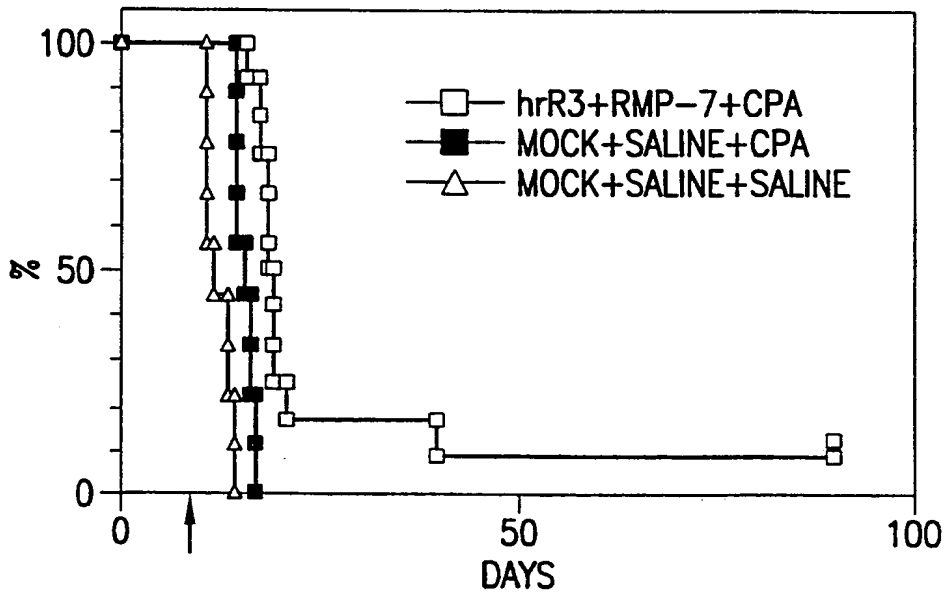


FIG. 2B

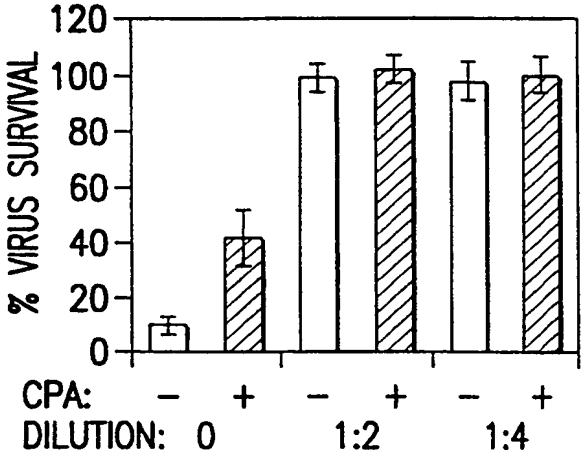


FIG. 3A

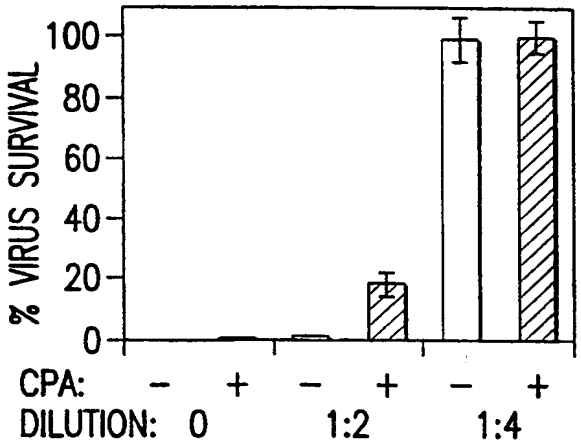


FIG.3B

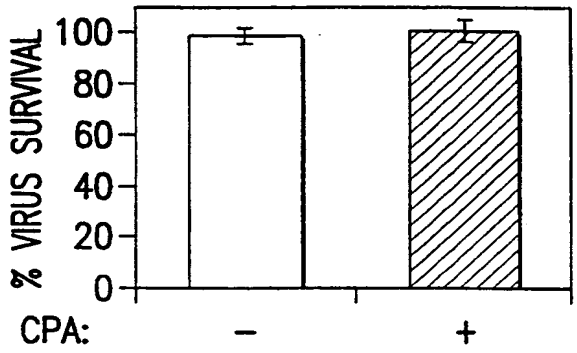


FIG.3C

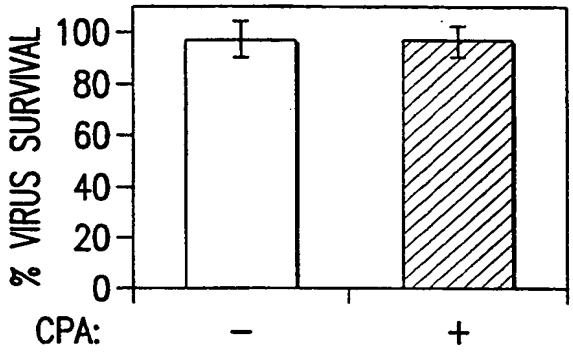
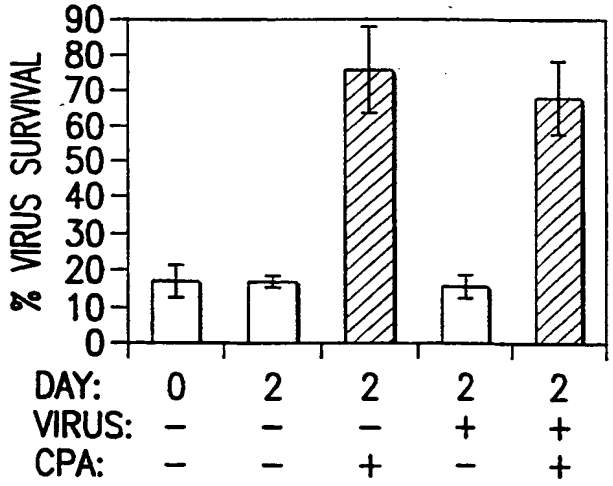


FIG.3D



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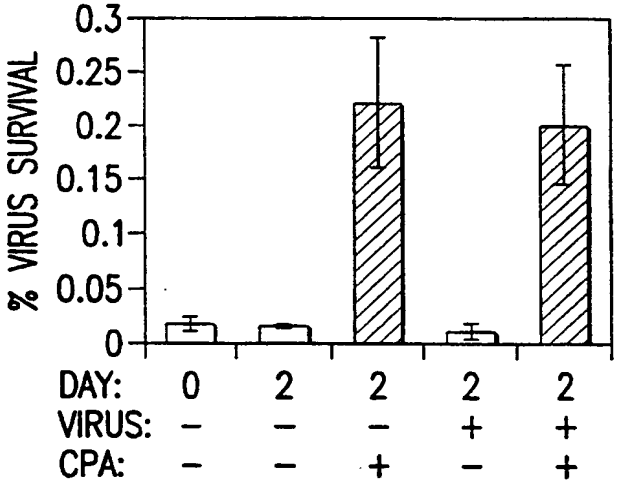

FIG.3F

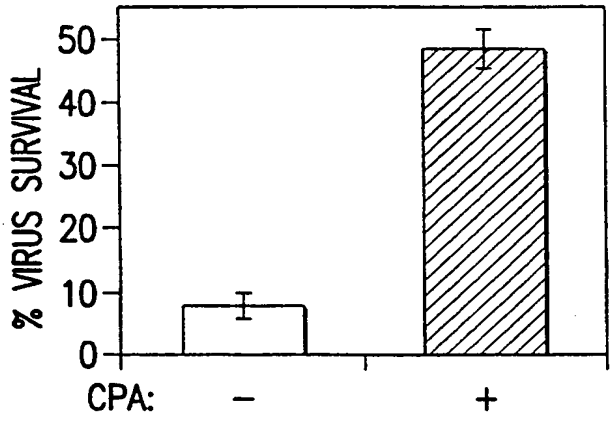

FIG.4A

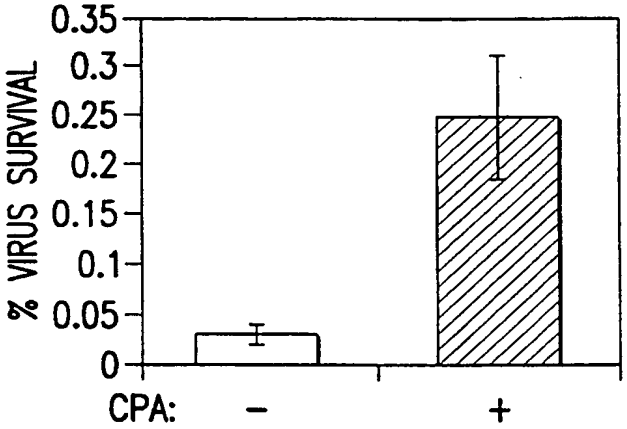

FIG.4B

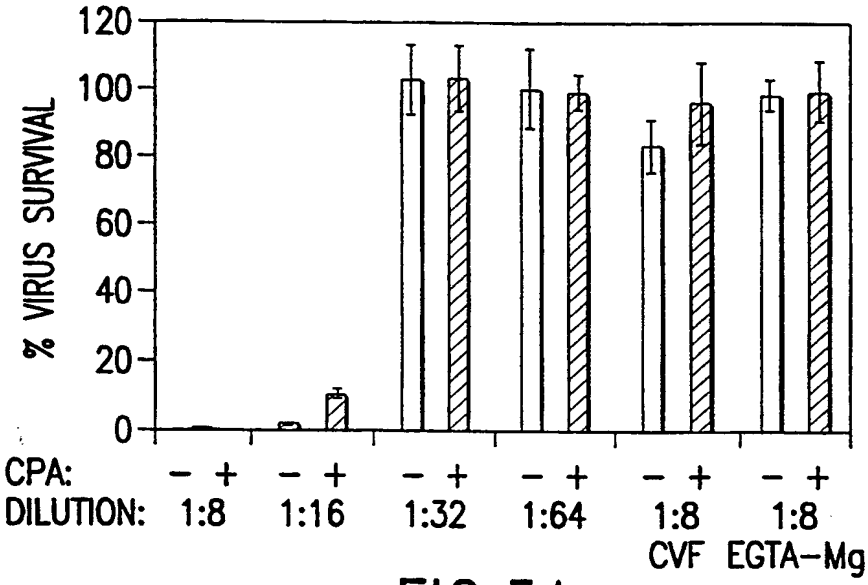

FIG.5A

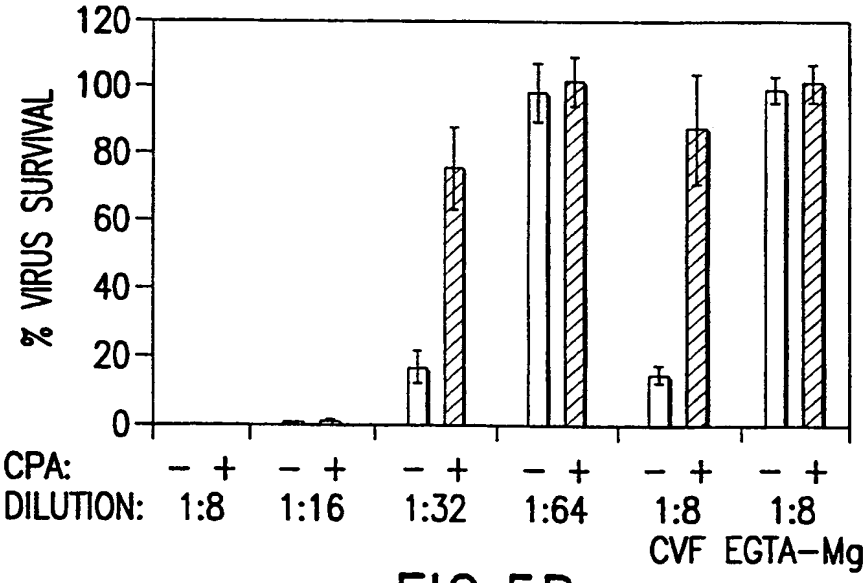

FIG.5B

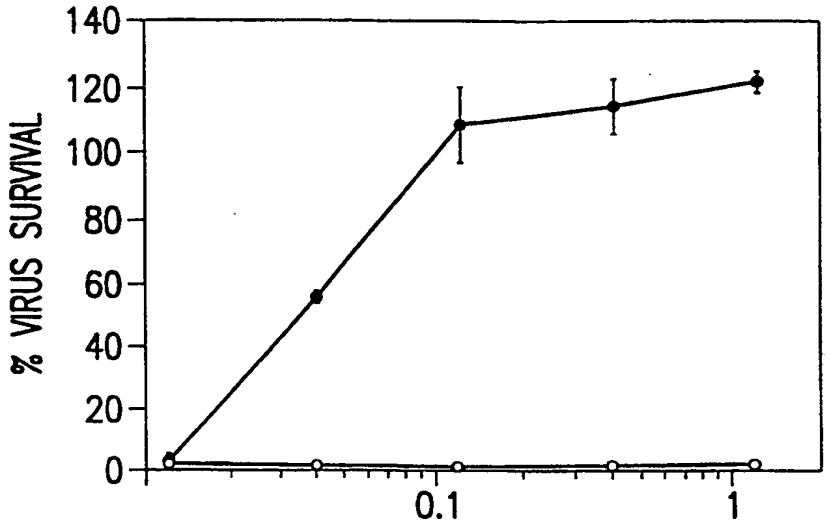

FIG.6A

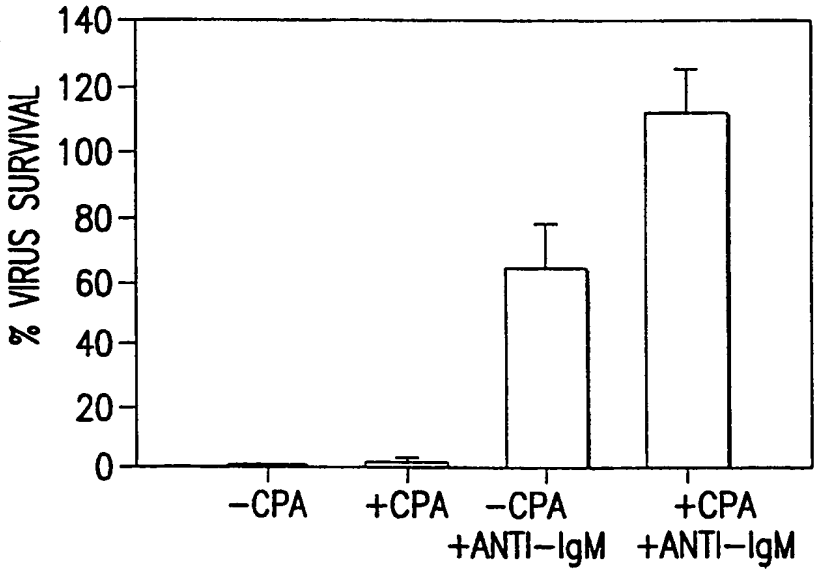

FIG.6B

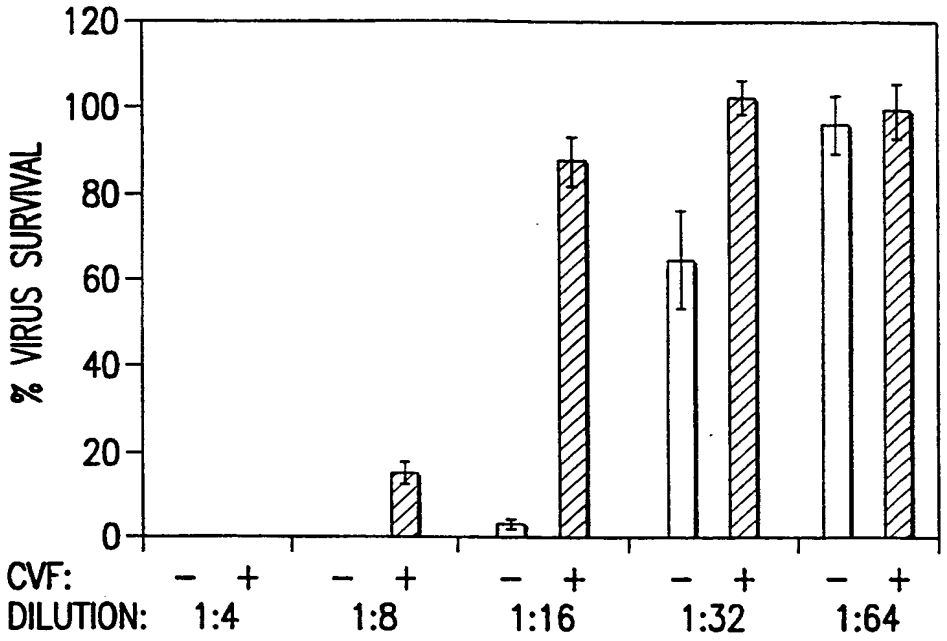

FIG.7

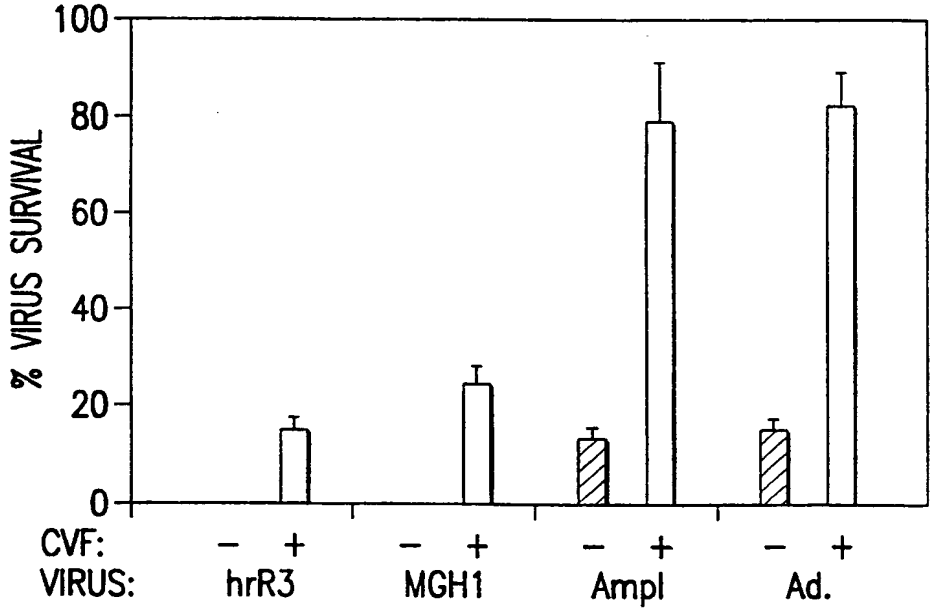

FIG.8

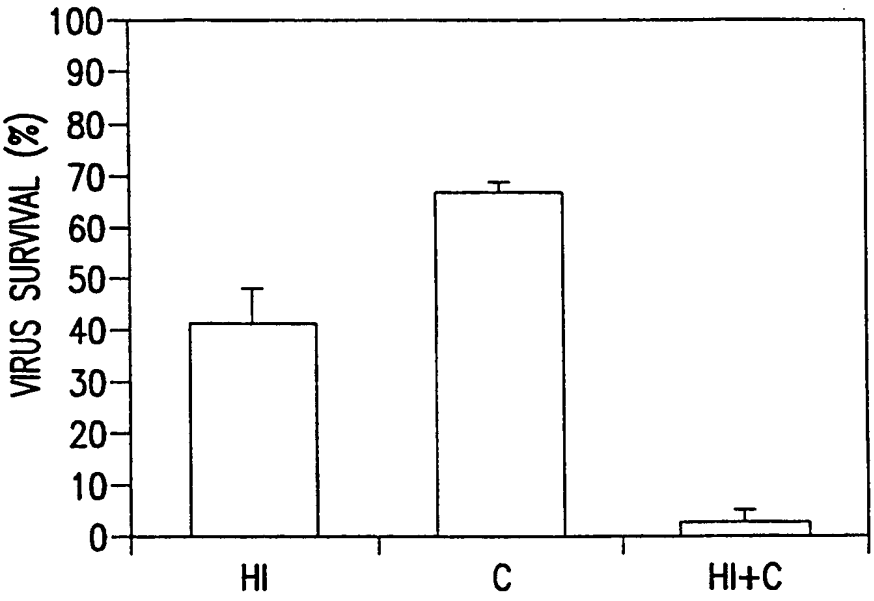

FIG.9

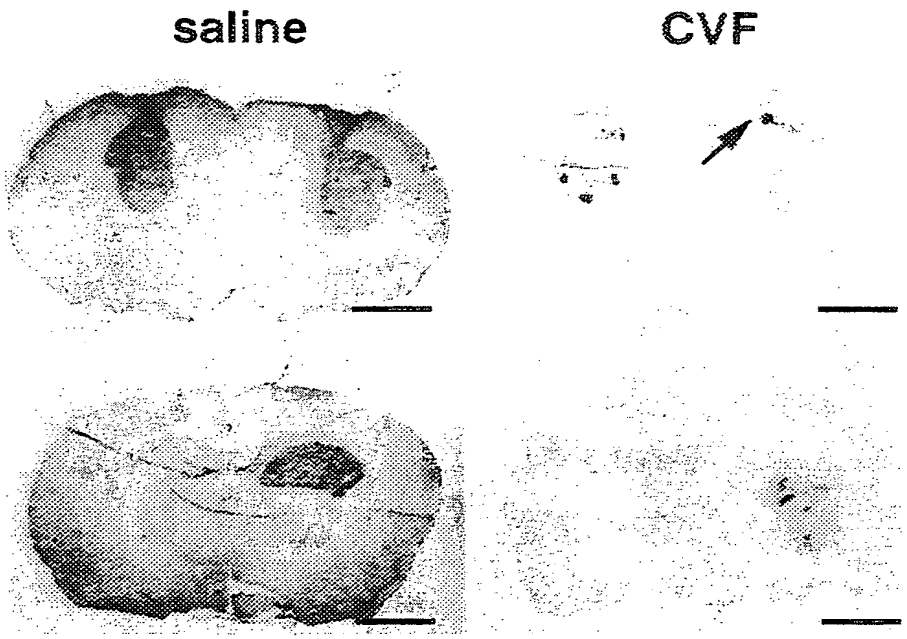

FIG. 10A

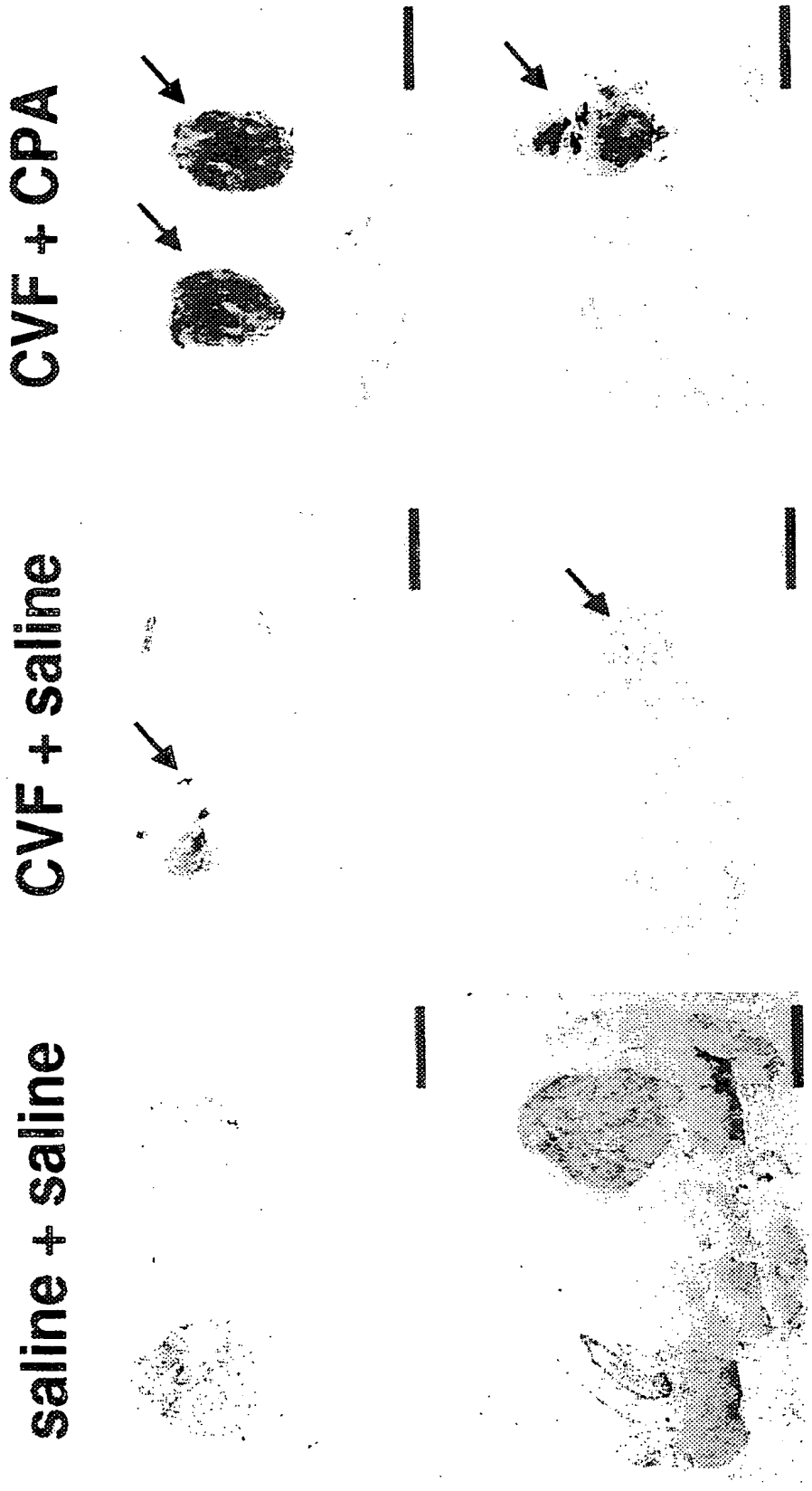

FIG.10B

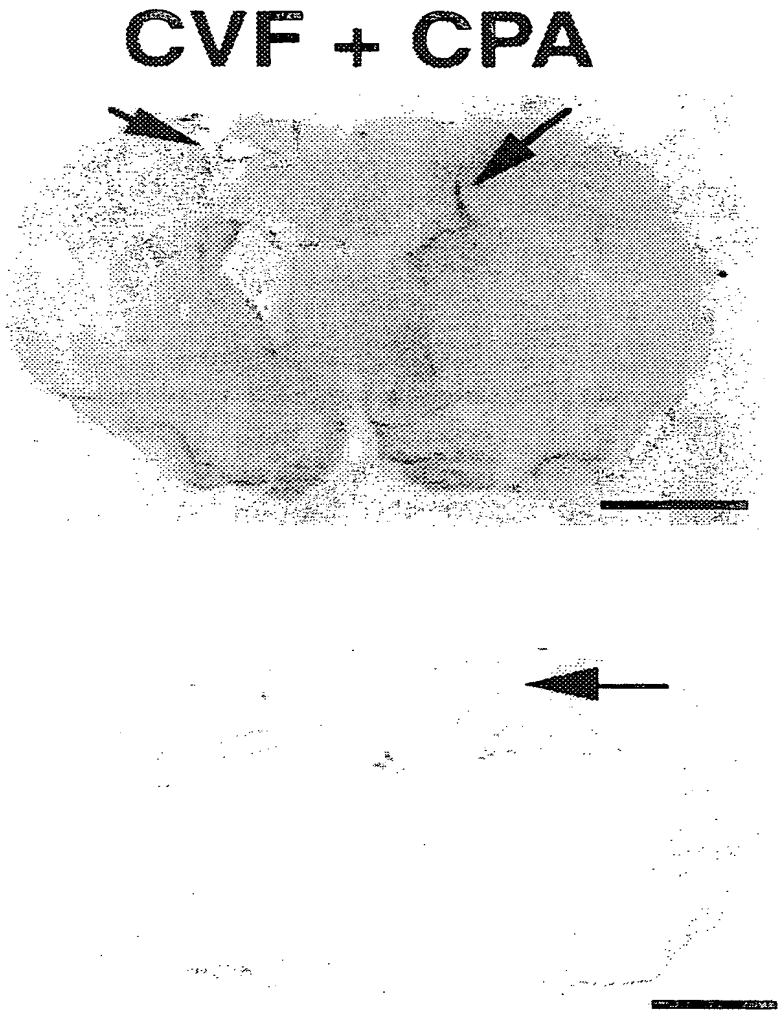

FIG. 10C

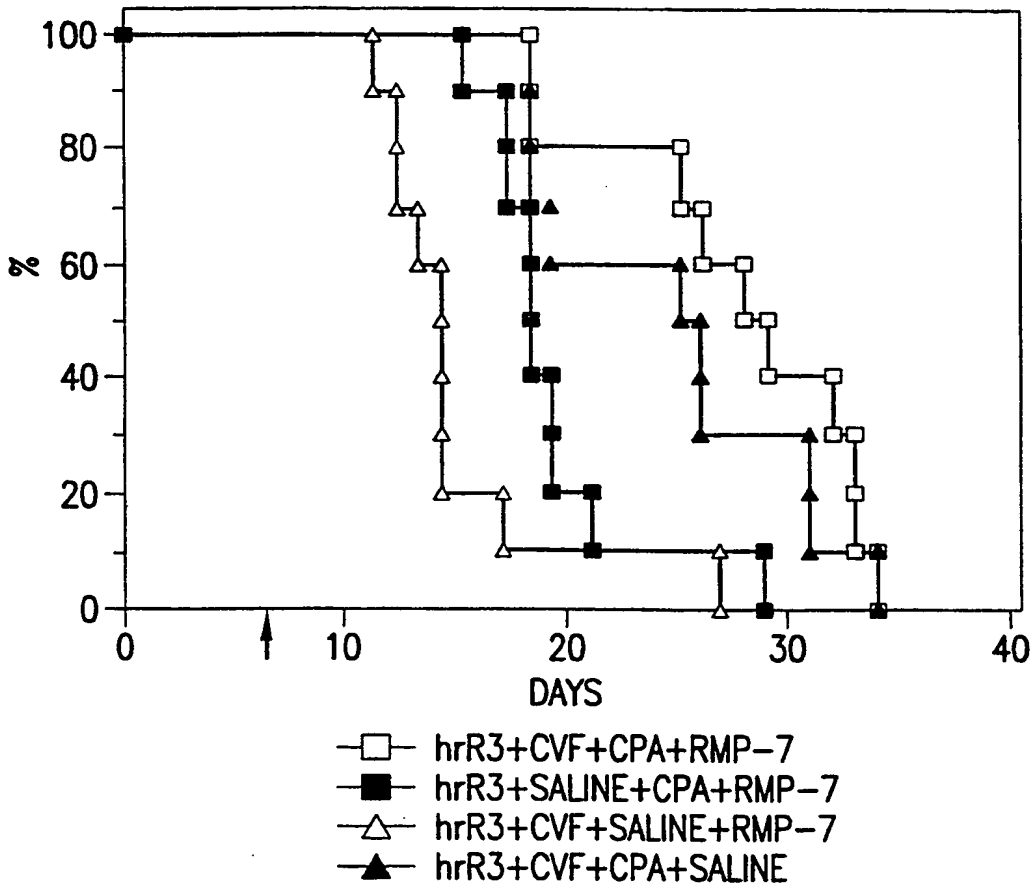

FIG. 11A

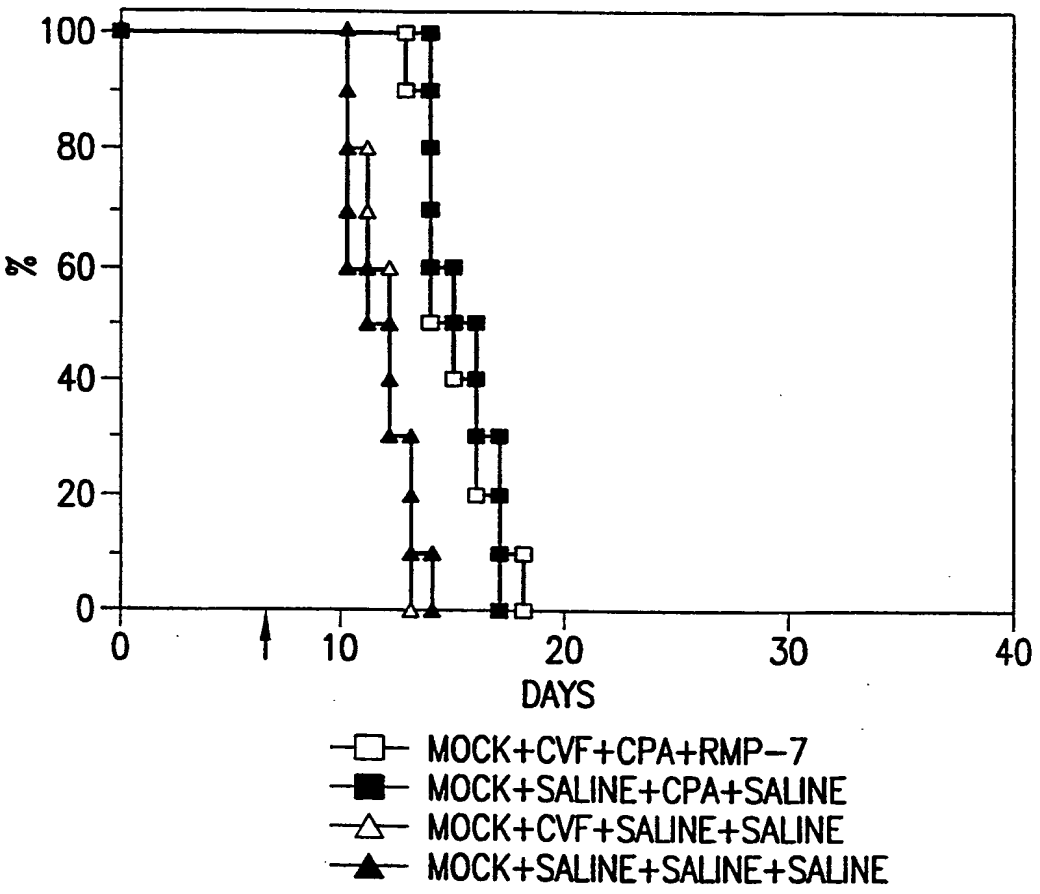

FIG.11B

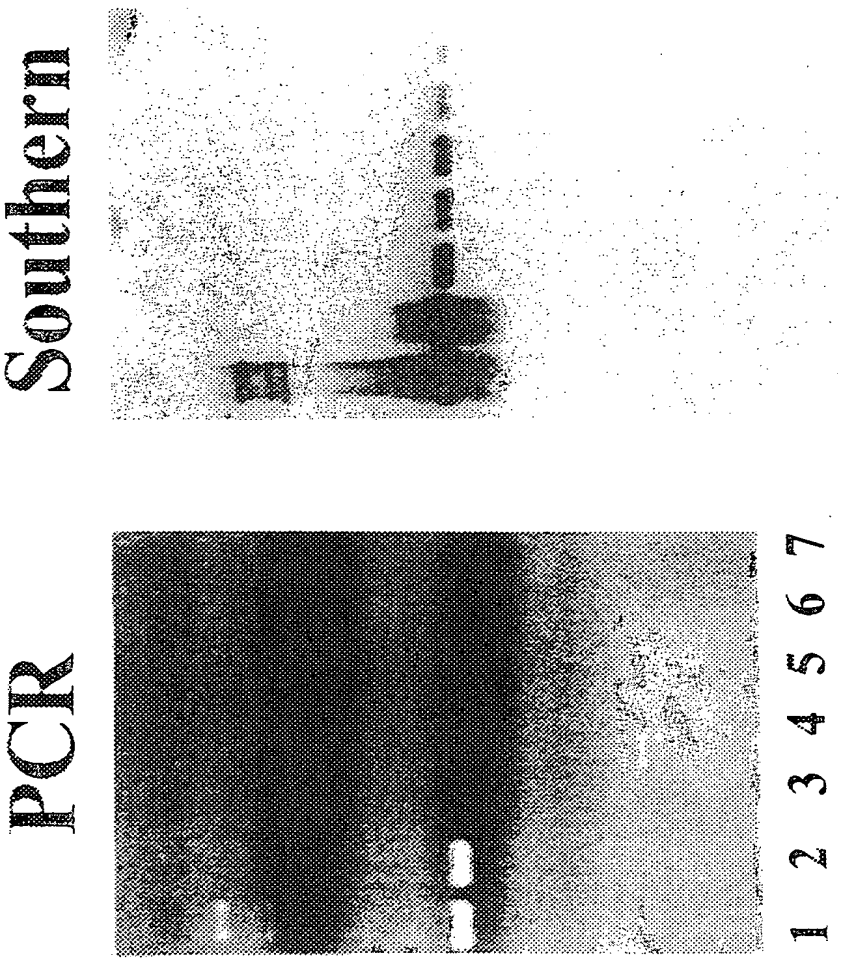

FIG.12

SEQUENCE LISTING

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Alkermes, Inc.
Chiocca, E. Antonio
Ikeda, Keiro
Bartus, Raymond T.

<120> A Method of Genetic Vector Delivery

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Statement Concerning Non-Prejudicial Disclosure or Exception to Lack of Novelty

Due to a public disclosure in August of 1999, the applicant respectfully requests that the subject international application be granted the respective provisions under national laws concerning Exceptions to Lack of Novelty in each of the designated countries. This is not an admission that the subject invention is not novel. Exception to Lack of Novelty is hereby requested for purposes of disclosure and precautionary measures.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 48/00, A61P 35/00

A3

(11) International Publication Number: **WO 00/29033**

(43) International Publication Date: 25 May 2000 (25.05.00)

(21) International Application Number: PCT/US99/27206

(22) International Filing Date: 17 November 1999 (17.11.99)

(30) Priority Data:
60/108,881 17 November 1998 (17.11.98) US

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(74) Agents: GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005–3934 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
With a statement concerning non-prejudicial disclosure or exception to lack of novelty.

(88) Date of publication of the international search report:
5 October 2000 (05.10.00)

(54) Title: A METHOD OF GENETIC VECTOR DELIVERY

(57) Abstract

The invention relates to a method of administering a genetic vector to a target cell in a patient by immunosuppressing the patient and administering the genetic vector. The invention also relates to a method of administering a genetic vector to a target cell in a patient by administering a complement inhibitor and the genetic vector. The invention also relates to further administering a blood–organ barrier modifier.

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SDOCID: <WO___0029033A3_I_>

INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/US 99/27206

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K48/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 12406 A (GENETIC THERAPY INC ;TRAPNELL BRUCE C (US); YEI SOONPIN (US); MCCL) 2 May 1996 (1996-05-02)	1,5,6, 11,12, 15,25, 28,29
Y	abstract page 1, paragraph 2 page 4, line 4 - line 14 page 6, line 23 -page 7, line 20 page 12, paragraph 2 page 13, paragraph 3 -page 18, paragraph 1; claims 1-4,12	2,7,9, 10,13, 14,26, 30,31

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-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 June 2000	27/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Niemann, F

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	BOUVET M ET AL: "Suppression of the immune response to an adenovirus vector and enhancement of intratumoral transgene expression by low-dose etoposide." GENE THERAPY FEB., 1998, vol. 5, no. 2, February 1998 (1998-02), pages 189-195, XP000907596 ISSN: 0969-7128	1,5,6, 11,12, 15,25, 28,29
Y	abstract page 189, left-hand column, line 1 -right-hand column, line 26 page 192, left-hand column, line 1 -right-hand column, line 45	2,7,9, 10,13, 14,26, 30,31
X	CHRIST MARIELLE ET AL: "Gene therapy with recombinant adenovirus vectors: Evaluation of the host immune response." IMMUNOLOGY LETTERS 1997, vol. 57, no. 1-3, 1997, pages 19-25, XP000907595 ISSN: 0165-2478	1,5,6, 11,12, 15,25, 28,29
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